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The Editor comments

ENLIGHTENED EMPIRICISM

"*Enlightened Empiricism*" is a rather descriptive phrase which has been applied of late to a number of various activities and disciplines. Generally, it has been done so in a complimentary manner, although in some few instances sensitive individuals or groups have regarded such reference to their own pursuits as being somewhat derogatory.

It is our belief that the term can be very properly applied to the present status of pharmacy as defined in its broad sense. In doing so, we consider such reference to be highly complimentary and one in which we all may take pride.

When one considers the rapid strides being made in the pharmaceutical field, even skeptics are forced to admit that pharmacy is in a state of transition in the course of which it is coming more and more to be a true and exact science. Our colleagues in other areas of activity are gradually coming to view us with greater esteem and to acknowledge our accomplishments. As a few examples, we might cite: (*a*) an understanding of structure-activity relationships is evolving which enables more accurate prediction of pharmacological properties based solely on a knowledge of chemical structure; (*b*) postulated absorption, excretion, and blood-level properties are possible because of data which have been accumulated regarding surface tension, dissociation constants, partition coefficients, and tendencies toward complex formation; (*c*) routes of drug degradation and detoxification, and liability of side reactions may be predicted with some accuracy through strides made in radioisotope work, advances in selective analytical methods, and elucidation of degradation kinetics; (*d*) renewed interest in plants as a source of useful drugs has resulted from a better knowledge of factors regulating growth and productivity, improved methods of extraction, and the development of unique and highly selective methods for isolating active principles.

These and other achievements are demonstrable evidence that pharmacy is very surely moving from the category of an art to a science. For some this will evoke feelings of nostalgia; for a few, perhaps this trend will meet with resistance. Such would indeed be unfortunate, however, and only serve as an obstacle in reaching the heights of acclaim which are possible. For surely as progress dictated that alchemy give way to modern science, so must our old concept of pharmacy, *the art*, pass its legacy on to pharmacy, *the science*. The old will not be lost in the new—rather it will serve as the foundation upon which the new is built.

Edward G. Feldmann

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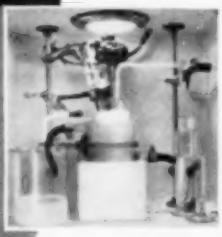

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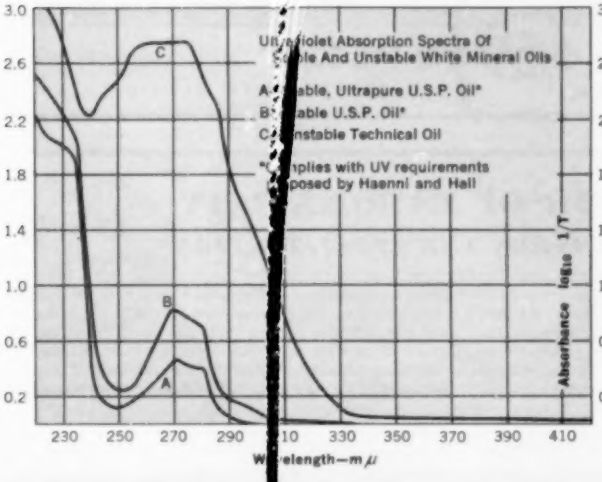

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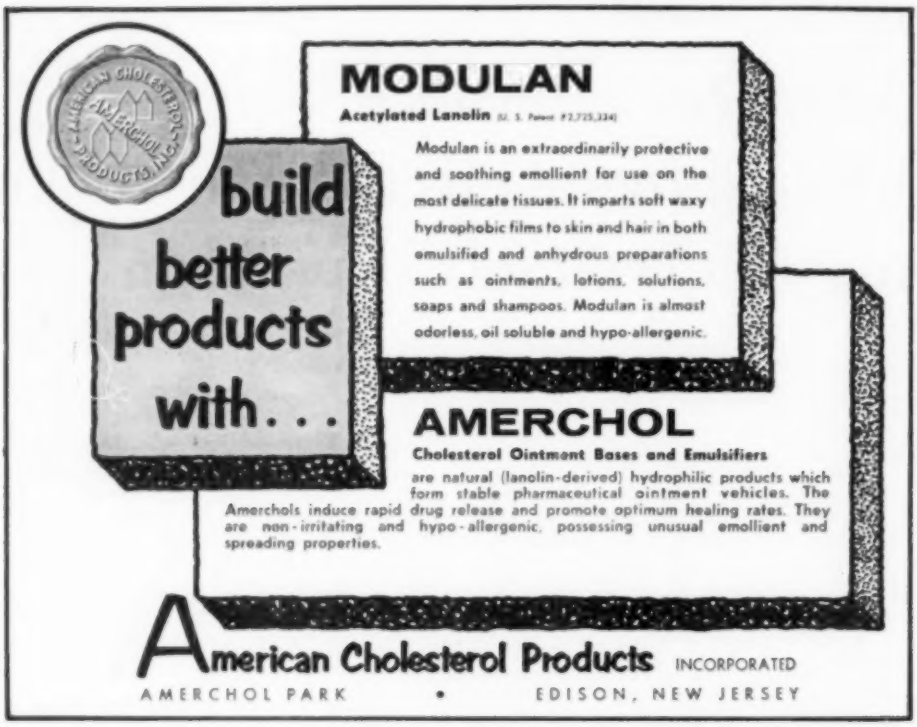
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JOURNAL OF **Pharmaceutical Sciences**

April 1961 volume 50, number 4

Review Article

Hypotensive Veratrum Ester Alkaloids

By S. MORRIS KUPCHAN

VERATRUM and related plants have been used for medicinal purposes for hundreds of years. Galenical preparations were used in the Middle Ages for purposes of sorcery and in mystical rites. Subsequently, the crude extracts have been used in the treatment of fevers, as local counterirritants in neuralgia, as cardiac tonics, as emetics, as crow poisons, and as insecticides (1, 2). The use of veratrum in the control of hypertension, at least in the United States, dates from the report of Baker in 1859 (3). Several attempts were made to introduce the use of veratrum extracts into medical practice during the second half of the nineteenth century, but these attempts were unsuccessful. The treatments during this period continued to employ crude extracts containing many alkaloids. The results achieved with these crude extracts were erratic and the treatments fell into disrepute. Some fifty years later, during the late 1930's, purified alkaloidal preparations responsible for the hypotensive activity of veratrum became available for the first time. Poethke in Germany (4) and Jacobs and Craig in the United States (5) improved the extraction and purification procedures and made available the first crystalline powerfully hypotensive alkaloid preparation, protoveratrine. Careful pharmacological investigation of the crystalline preparations, spearheaded by Krayer and his associates, demonstrated that the drugs were suitable for clinical trials (6). These clinical trials were followed by introduction of protoveratrine and related preparations into clinical use in the treatment of certain types of hypertension (7–9).

The principal hypotensive principles of veratrum extracts are known to be ester derivatives of four highly hydroxylated native veratrum alkamines.[1] The chemistry of the alkamines zygadenine, veracevine, and germine has been summarized in several reviews (13–15). The elucidation of structure and configuration of protoverine was recently discussed in detail (16). Studies of the hypotensive ester alkaloids of the series, on the other hand, have not been the focus of any published review. In a recently completed paper, the occurrence of ester alkaloids and taxonomy of veratrum and related genera were surveyed (17). The present account summarizes the results of structural investigations and discusses apparent relationships between structure and hypotensive activity among the hypotensive veratrum ester alkaloids.

The ceveratrum alkamines occur in nature as

This is part XLVII of a series entitled "Veratrum Alkaloids;" part XLVI, Kupchan, S. M., Grivas, J. C., Ayres C. I., Pandya, L. J., and Weaver, L. C., THIS JOURNAL, in press.

Received from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison.

[1] Sabine, $C_{29}H_{45}NO_7$, and its monoacetate ester, sabadine, $C_{31}H_{47}NO_8$, will merit inclusion in a future review of this type. Omission from this paper was deemed desirable because of the incompleteness of the structural studies on the latter compounds (10–12).

TABLE I.—STRUCTURES OF THE HYPOTENSIVE VERATRUM ESTER ALKALOIDS

Compound	Formula	Acyl Groups[a] C_3	C_6	C_7	C_{16}	References
Esters of Zygadenine						
Zygacine (I)	$C_{29}H_{45}NO_8$	Ac	...	...	...	(18, 19)
Angeloylzygadenine (V)	$C_{32}H_{49}NO_8$	An	...	...	...	(20)
Veratroylzygadenine (XII)	$C_{36}H_{51}NO_{10}$	Ve	...	...	...	(21, 22)
Vanilloylzygadenine (XIV)	$C_{36}H_{49}NO_{10}$	Va	...	...	...	(21)
Esters of Veracevine						
Cevacine (XVIII)	$C_{29}H_{45}NO_9$	Ac	...	...	...	(23, 24)
Cevadine (XXII)	$C_{32}H_{49}NO_9$	An	...	...	...	(25–27)
Veratridine (XXVI)	$C_{36}H_{51}NO_{11}$	Ve	...	...	...	(23–26)
Vanilloylveracevine[b] (XXVII)	$C_{36}H_{49}NO_{11}$	Va	...	...	...	(28)
Esters of Germine						
Germitetrine (XXVIII)	$C_{41}H_{61}NO_{14}$	HMAB	...	Ac	MB	(29–31)
Germitrine (XXXV)	$C_{39}H_{61}NO_{12}$	MB	...	Ac	HMB	(32, 33)
Neogermitrine (XXXIX)	$C_{39}H_{59}NO_{11}$	Ac	...	Ac	MB	(33, 34)
Germanitrine (XLIV)	$C_{39}H_{59}NO_{11}$	An	...	Ac	MB	(35, 36)
Germinitrine[c]	$C_{39}H_{57}NO_{11}$					(35)
Germerine (XXXVI)	$C_{37}H_{59}NO_{11}$	MB	...	...	HMB	(4, 33, 37)
Germidine (XL)	$C_{31}H_{51}NO_{10}$	Ac	...	...	MB	(32, 33)
Neogermidine[d] (XLI)	$C_{31}H_{51}NO_{10}$	...	...	Ac	MB	(33, 38, 39)
Germbudine (XLVII)	$C_{37}H_{59}NO_{12}$	*t*-DMB	...	...	MB	(39, 40)
Neogermbudine (XXXII)	$C_{37}H_{59}NO_{12}$	*e*-DMB	...	...	MB	(30, 31)
Protoveratridine (XXXVII)	$C_{28}H_{41}NO_8$	MB	...	...	...	(4, 33, 37)
Esters of Protoverine						
Protoveratrine A[e] (XLIX)	$C_{41}H_{63}NO_{14}$	HMB	Ac	Ac	MB	(29, 30, 41–43)
Protoveratrine B[f] (LXII)	$C_{41}H_{63}NO_{15}$	*t*-DMB	Ac	Ac	MB	(29, 30, 41–43)
Escholerine (LXVIII)	$C_{41}H_{61}NO_{13}$	An	Ac	Ac	MB	(44, 45)
Desacetylprotoveratrine A (LXIX)	$C_{33}H_{51}NO_{13}$	HMB	Ac	...	MB	(30, 46)
Desacetylprotoveratrine B[g] (LXXII)	$C_{35}H_{51}NO_{14}$	*t*-DMB	Ac	...	MB	(39, 46, 47)

[a] Ac = acetyl; An = angeloyl; Ve = veratroyl; Va = vanilloyl; HMAB = *erythro*-2-hydroxy-2-methyl-3-acetoxybutyryl; MB = (*l*)-2-methylbutyryl; HMB = (*d*)-2-hydroxy-2-methylbutyryl; *t*-DMB = (*d*)-*threo*-2,3-dihydroxy-2-methylbutyryl; *e*-DMB = (*l*)-*erythro*-2,3-dihydroxy-2-methylbutyryl. [b] Also called vanilloylcevine. [c] Germinitrine has been reported to be a triester of germine which yields acetic acid, tiglic acid and angelic acid upon hydrolysis (35). The positions of the acyl groups have not been determined. [d] Also called isogermidine. [e] Also called protoveratrine. [f] Also called neoprotoveratrine and veratetrine. [g] Also called desacetylneoprotoveratrine.

esters in combination with from one to four molecules of acids. Aforementioned reviews have tabulated the sources (17) and properties (13, 15) of the ester alkaloids. Table I summarizes the results of the recent studies on the structures of the esters, and the manner in which these conclusions were drawn constitutes the principal subject of the further discussion.

Zygacine (I) was characterized as a monoacetate ester of zygadenine (II) by the fact that methanolysis of zygacine yielded zygadenine and acetic acid (after alkaline hydrolysis of the

volatile methanolysis products) (18). Zygacine formed an acetonide, assigned the 14,15-acetonide structure (III). The fact that zygacine (I) consumed two molar equivalents of periodic acid whereas the acetonide (IV) consumed none indicated that the acetate group of zygacine blocks cleavage of the ring A glycol; i. e., that the acetate group is located either at C_3 or C_4. A zygadenine 4-monoacetate on acetylation with acetic anhydride would form zygadenine 3,4,-15,16-tetraacetate (19). In fact, zygacine yielded zygadenine 3,15,16-triacetate upon acetylation under the aforementioned conditions, and zygacine was therefore assigned the zygadenine 3-monoacetate structure (I) (19).

Angeloylzygadenine (V) was shown to be a monoangelate ester of zygadenine by alkaline hydrolysis to isozygadenine (VI) and angelic acid (VII). The characterization was confirmed by transformation of V under more vigorous alkaline treatment to pseudozygadenine (VIII) and tiglic acid (IX). Angeloylzygadenine consumed two mole equivalents of periodic acid to yield a crystalline cyclopentanonealdehyde (X), and the formation of the latter product is indicative of the fact that the angelate group is located at either C_3 or C_4. Acetylation of angeloylzygadenine yielded triester XI, and the naturally occurring monoester was therefore assigned the zygadenine 3-monoangelate structure (V) (20).

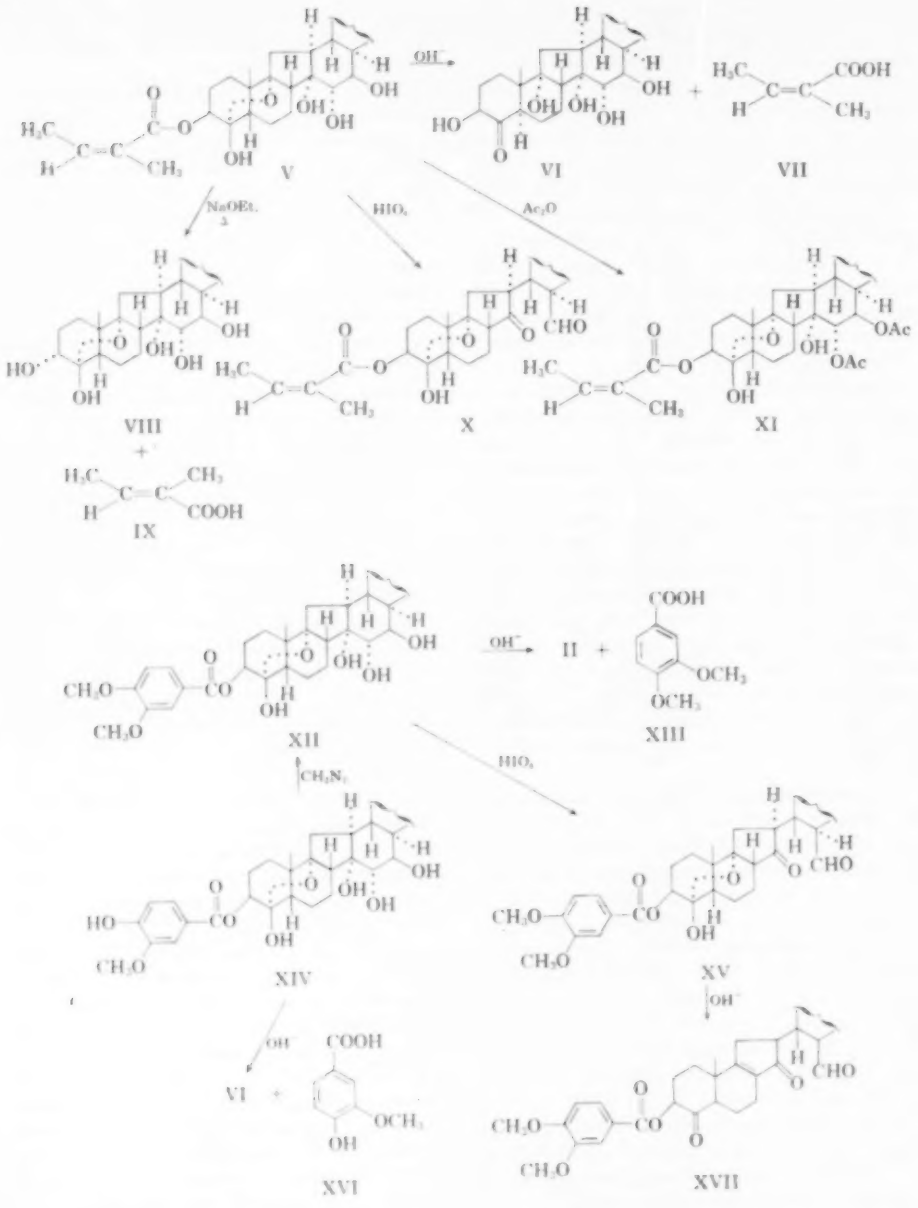
Veratroylzygadenine (XII) was first cleaved by alkaline hydrolysis to isozygadenine (VI) and veratric acid (XIII) (21). Stronger alkaline treatment yielded pseudozygadenine (VIII) (21); hydrolysis in the cold gave zygadenine (II) (22). Veratroylzygadenine consumed two mole equivalents of periodic acid to yield a product which showed absorption in the infrared indicative of structure XV (e. g., band at 5.75 μ for the cyclopentanone). Exposure of the periodic acid cleavage product to alkali for ten minutes gave rise to a product which showed intense absorption at 238 mμ[2] as required for XVII (48, *cf.* 19). The latter facts made possible exclusion of sites other than C_3 or C_4 as points of attachment of the veratroyl residue. Assignment to C_3 is strongly favored by biogenetic analogy to the acetate (I) and angelate (V) esters. Furthermore, no C_4 ester has been found among the multitude of naturally occurring ester derivatives listed in Table I. Vanilloylzygadenine was characterized by alkaline hydrolysis to isozygadenine (VI) and vanillic acid (XVI). Assignment of structure XIV is based upon the conversion to veratroylzygadenine upon methylation with diazomethane (21).

Cevacine (XVIII) was first characterized as a monoacetate ester of veracevine by methanolysis to veracevine (XX) and by acetylation of cevacine to veracevine 3,4,16-triacetate (XIX) (23). Subsequent partial synthesis of cevacine by partial acetylation of veracevine confirmed the fact that veracevine is the true alkamine of cevacine (24). The lead tetraacetate consumption of cevacine [one mole equivalent (24)] indicated that the acetate ester group blocks the ring A glycol and is therefore affixed at either C_3 or C_4. Assignment to C_3 is strongly favored by biogenetic analogy to cevadine and to the zygadenine monoesters.

Cevadine (XXII) is a monoester which yields two labile components upon hydrolysis. As a result, cevadine has been regarded at various times as containing as alkanolamine either cevine, cevagenine, or veracevine (23, 25, 26); the acid moiety has been regarded as either tiglic (IX) or angelic acid (VII). The mildest hydrolytic conditions afforded veracevine and angelic acid and made the latter appear to be the most likely component parts of cevadine (23, 25, 26). That veracevine and angelic acid are indeed the true components of the natural ester alkaloid was established by the synthesis from veracevine of an angelate ester identical with veracevine (27). Veracevine was treated with a limited amount of 3-bromoangeloyl chloride to yield veracevine 3-(3′-bromoangelate) (XXI). The latter compound on hydrogenolysis afforded cevadine (XXII). That the angelate residue blocks the C_3, C_4 α-ketol hemiketal system was established by periodic acid oxidation (consumption: one mole equivalent) to a product which was not a γ-lactone like the product from a cevine derivative with a free α-ketol hemiketal system. Evidence in support of the C_3 ester structure (favored on biogenetic grounds) was secured by chromic acid titration experiments. Cevadine D-orthoacetate (XXIII) demonstrated a chromic acid consumption which paralleled that of cevadine D-orthoacetate monoacetate (XXIV) and indicated the presence of one secondary hydroxyl group. C_4 was therefore excluded as a site for attachment of the angelate ester of cevadine, for a C_4-attachment would leave two chromic acid-sensitive secondary hydroxyl groups in cevadine D-orthoacetate (27). The combination of the results discussed strongly support the veracevine 3-angelate structure (XXII) for cevadine.

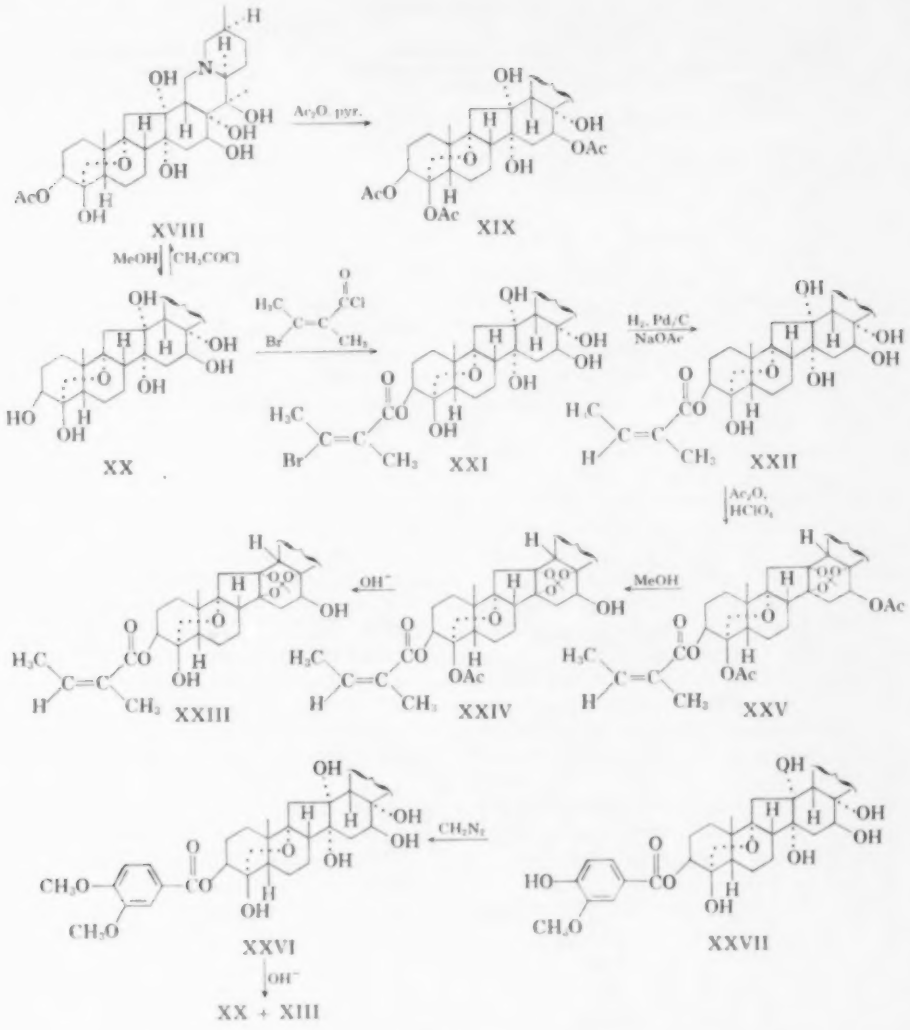
Veratridine (XXVI) was first assigned a

[2] Compensated for aromatic ester absorption by using an equivalent amount of veratroylzygadenine in the alcohol blank.

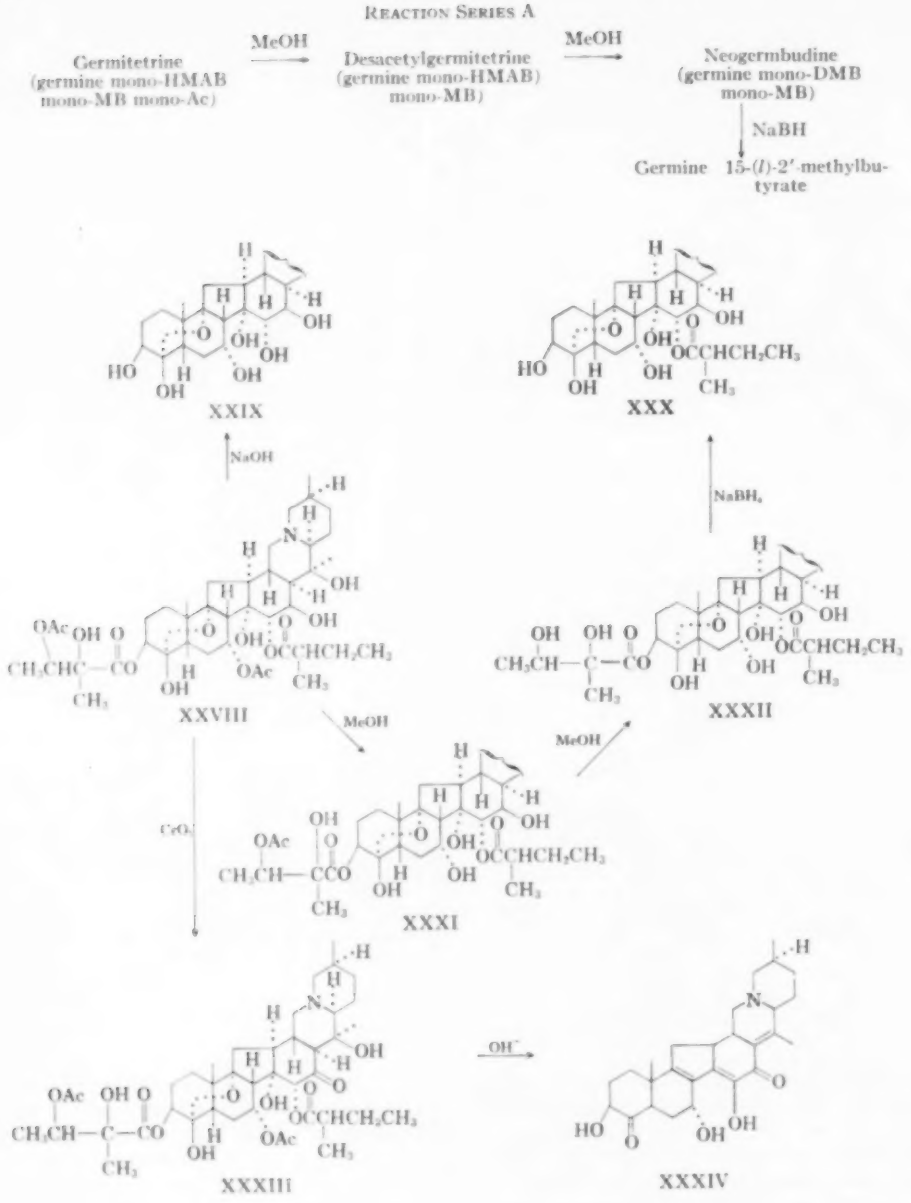


veratroylveracevine constitution on the basis of alkaline hydrolysis to veracevine (XX) and veratric acid (XIII) (23, 26). Subsequent partial synthesis by acylation of veracevine with veratroyl chloride confirmed the fact that veracevine is the true alkamine of veratridine (24). Assignment of C_3 as the point of attachment of the veratroyl residue is strongly favored on the basis of biogenetic analogy to cevadine Vanilloylveracevine was characterized by alkaline hydrolysis to cevine and vanillic acid (XVI). Assignment of structure XXVII is based upon the conversion to veratridine upon methylation with diazomethane (28).

Germitetrine (XXVIII) was characterized as a tetraester of germine (XXIX) (49) by hydrolysis

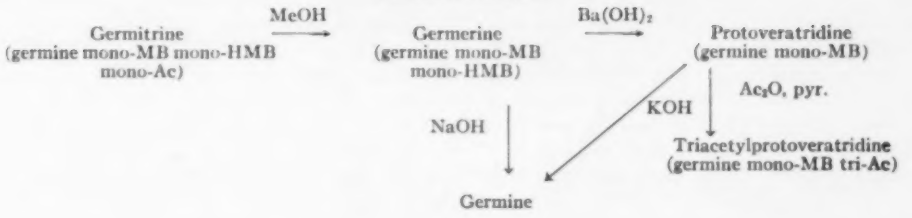


to germine and two mole equivalents of acetic acid, one mole equivalent of (*l*)-2-methylbutyric acid, and one mole equivalent of (*l*)-*erythro*-2,3-dihydroxy-2-methylbutryic acid (29, 30). Methanolysis of germitetrine was shown to yield two degradation products; loss of one acetate grouping led to desacetylgermitetrine, whereas removal of both acetate groupings resulted in conversion to the naturally occurring germine diester, neogermbudine (30) (Reaction series A). The acetate grouping methanolyzed in the conversion of germitetrine to desacetylgermitetrine was first provisionally assigned to C_7 on the basis of molecular rotation differences and the characteristically facile methanolysis of C_7 acetates (33). Next, the four acyl groups of germitetrine were tentatively assigned to positions C_3, C_7, C_{15}, and C_{16} of germine (13). That the latter hypothesis was untenable was demonstrated by the observation that germitetrine was completely stable toward sodium periodate. The proposed tetraester would have a free glycol present in its dihydroxymethylbutyryl residue and would therefore be readily attacked by sodium periodate. Furthermore, germitetrine was shown to possess a free C_{16} hydroxyl group in the following manner. Chromic acid titration showed the presence of only one secondary hydroxyl group. The oxidation product, germitetrone (XXXIII), on alkaline hydrolysis gave the diosphenol



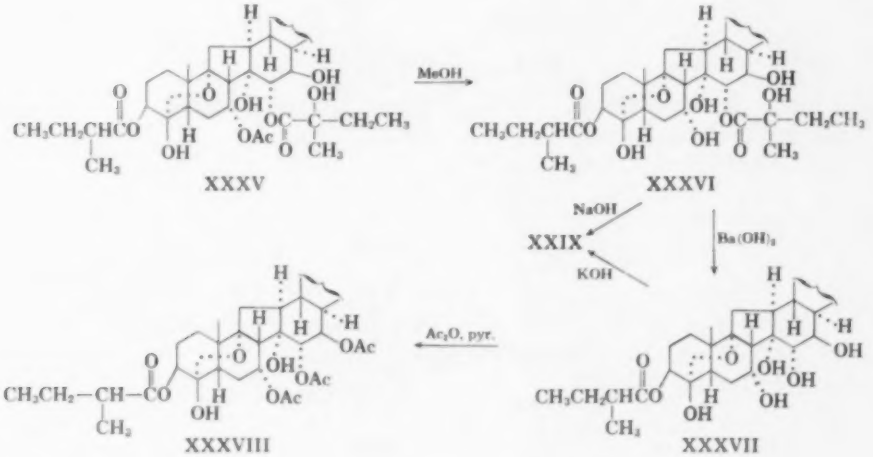
(XXXIV) previously obtained from neogermitrone and 16-dehydrogermine 3,4,7,15-tetraacetate (33). Thus germitetrine was shown to be a germine 3,7,15-triester in which the secondary hydroxyl group present in the dihydroxymethylbutyryl residue is acylated, thereby accounting for the four mole equivalents of acid produced upon hydrolysis. The location of germitetrine's more easily methanolyzed acetyl residue at C_7 required that desacetylgermitetrine be a germine 3,15-diester in which the secondary hydroxyl group of the dihydroxymethylbutyryl residue is acylated. The remaining structural questions were settled by studies on neogermbudine (XXXII), the germine mono-(*l*)-2′-methylbutyrate mono-2′,3′-dihydroxy-2′-methyl-

REACTION SERIES B

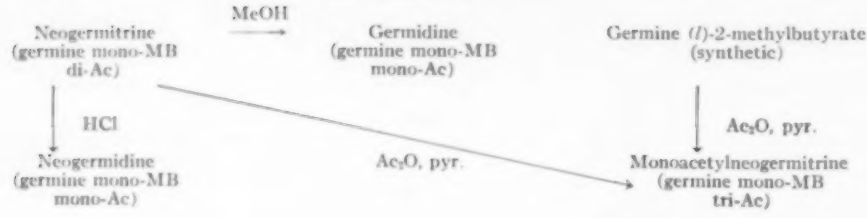


butyrate which is obtained by methanolysis of desacetylgermitetrine (see Reaction series A). Neogermbudine consumed one mole equivalent of sodium periodate in agreement with assignment of the acyl residues at C_3 and C_{15}. The specific location of each substituted butyryl residue was determined by use of the selectivity of the sodium borohydride reduction of esters of hydroxyacids. When neogermbudine was treated with sodium borohydride, the dihydroxymethylbutyryl ester grouping was selectively removed and germine 15-(*l*)-2′-methylbutyrate (XXX) was isolated. The foregoing experiments established that neogermbudine is germine 3-(*l*)-*erythro*-2′,3′-dihydroxy-2′-methylbutyrate 15-(*l*)-2′-methylbutyrate (XXXII), desacetylgermitetrine is germine 3-*erythro*-2′-hydroxy-2′-methyl-3′-acetoxybutyrate 15-(*l*)-2′-methylbutyrate (XXXI), and germitetrine is germine 3-*erythro*-2′-hydroxy-2′-methyl-3′-acetoxybutyrate 7-acetate 15-(*l*)-2′-methylbutyrate (XXVIII) (31).

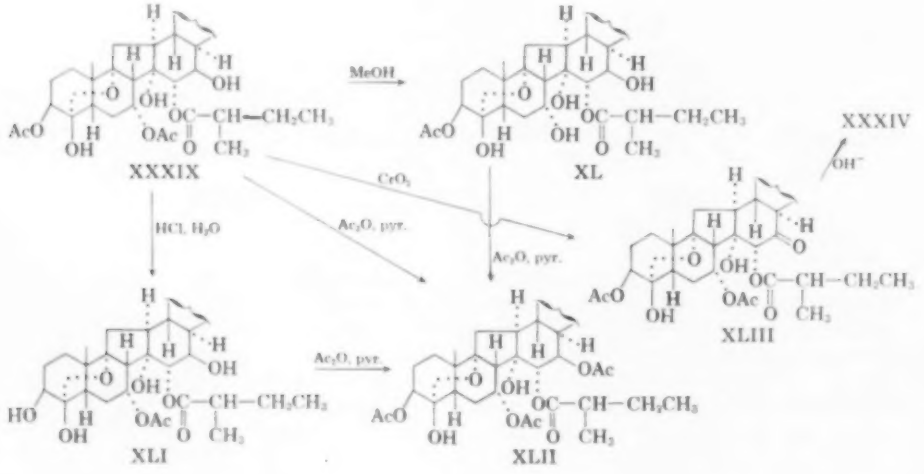
Germitrine (XXXV) is a naturally occurring triester which was converted by methanolysis to the diester germerine (32). Alkaline hydrolytic cleavage of germerine gave germine, (*l*)-2-methylbutyric acid, and (*d*)-2-hydroxy-2-methylbutyric acid. Partial hydrolysis of germerine with barium hydroxide afforded the monoester protoveratridine (XXXVII). Hydrolysis of protoveratridine with methanolic potassium hydroxide yielded germine (see Reaction series B). Germerine was found to consume periodic acid at a negligible rate, an indication that germerine contains an ester group on a hydroxyl group of the ring A glycol and a second ester group at C_{15} (33). Since protoveratridine is isomeric with germine 15-(*l*)-2′-methylbutyrate (XXX) (38), it is the (*l*)-2-methylbutyrate residue which is located in ring A, i.e., at C_3 or C_4. A germine 4-monoester on nonforcing acetylation would form a pentaester (48). In fact, protoveratridine is known to form a tetraester on acetylation, and a germine 4-(*l*)-2′-methylbutyrate structure is therefore precluded. Hence protoveratridine is germine 3-(*l*)-2′-methylbutyrate (XXXVII) and germerine consequently has structure XXXVI. The acetate group of germitrine shows the same reactivity as the C_7 acetate of neogermitrine and a strikingly similar contribution to the molecular rotation. On this basis, the acetate of germitrine is assigned to C_7, leading to the germine 3-(*l*)-2′-methylbutyrate 7-acetate 15-(*d*)-2′-hydroxy-2′-methylbutyrate structure (XXXV) for germitrine.



REACTION SERIES C



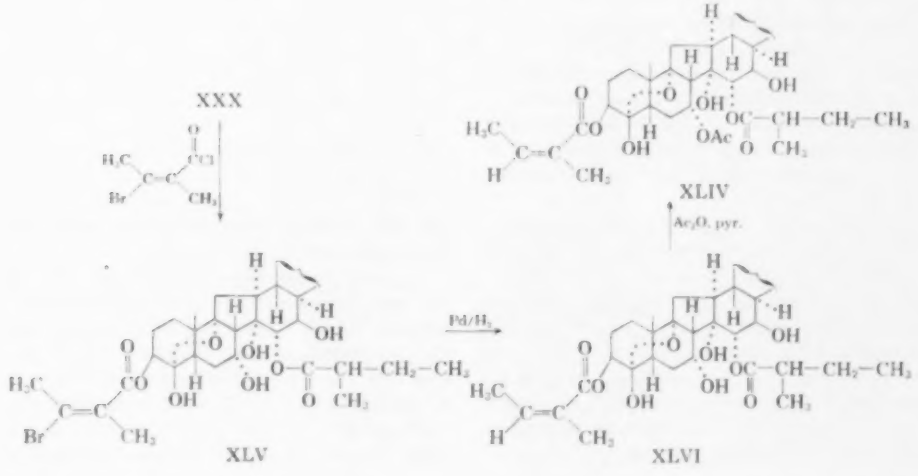
The naturally occurring triester neogermitrine (XXXIX) was shown to undergo methanolysis resulting in the loss of one acetate grouping with conversion to the diester germidine (32). Acid hydrolysis of neogermitrine led to loss of an acetate group with conversion to the diester neogermidine (33). Neogermitrine, germidine, neogermidine, and a synthetic mono-(*l*)-2-methylbutyrate of germine were shown to be convertible to the same acetylation product, monoacetylneogermitrine (see Reaction series C). These relationships revealed that the site of attachment of the (*l*)-2-methylbutyryl residue is the same in each of the four precursors of monoacetylneogermitrine. Periodic acid oxidation studies showed the site of attachment of the 2-methylbutyryl residue. Germidine and neogermitrine were stable to periodic acid; neogermidine consumed one mole equivalent of periodic acid and yielded an aldehydo-γ-lactone characteristic of cleavage of the ring A α-ketol hemiketal system. These results showed that neogermidine contains no ester functions in ring A, whereas germidine contains an ester group on a hydroxyl group of the ring A glycol and a second ester group at C_{15}. These facts and the aforementioned conclusion that the location of the 2-methylbutyryl residue is the same in these alkaloids fixed the site of attachment of the residue at C_{15} (33). The same conclusion was reached from studies on the periodate oxidation of the germine mono-(*l*)-2-methylbutyrate (XXX) (31). Since monoacetylneogermitrine can be obtained by stepwise acylation of germine (50), it appeared reasonable to assume that the same hydroxyl groups are acylated as in the analogous germine 3,7,15,16-tetraacetate (49). The acetate group of germidine therefore is located at C_3 and germidine was assigned structure XL. The second acetate of neogermitrine was assigned to C_7 (leading to expression XXXIX for neogermitrine, and, consequently, expression XLI for neogermidine) on the basis of the following sequence. Oxidation of neogermitrine with chromic anhydride-acetic acid yielded neogermitrone, formulated as the dehydro derivative XLIII. Alkaline treatment of neogermitrone yielded a diosphenol assigned structure XXXIV on the basis of its spectral properties and the fact that it could be obtained also from 16-dehydrogermine 3,4,7,15-tetraacetate (33). Thus, neogermitrine is germine 3,7-diacetate 15-(*l*)-2′-methylbutyrate (XXXIX),

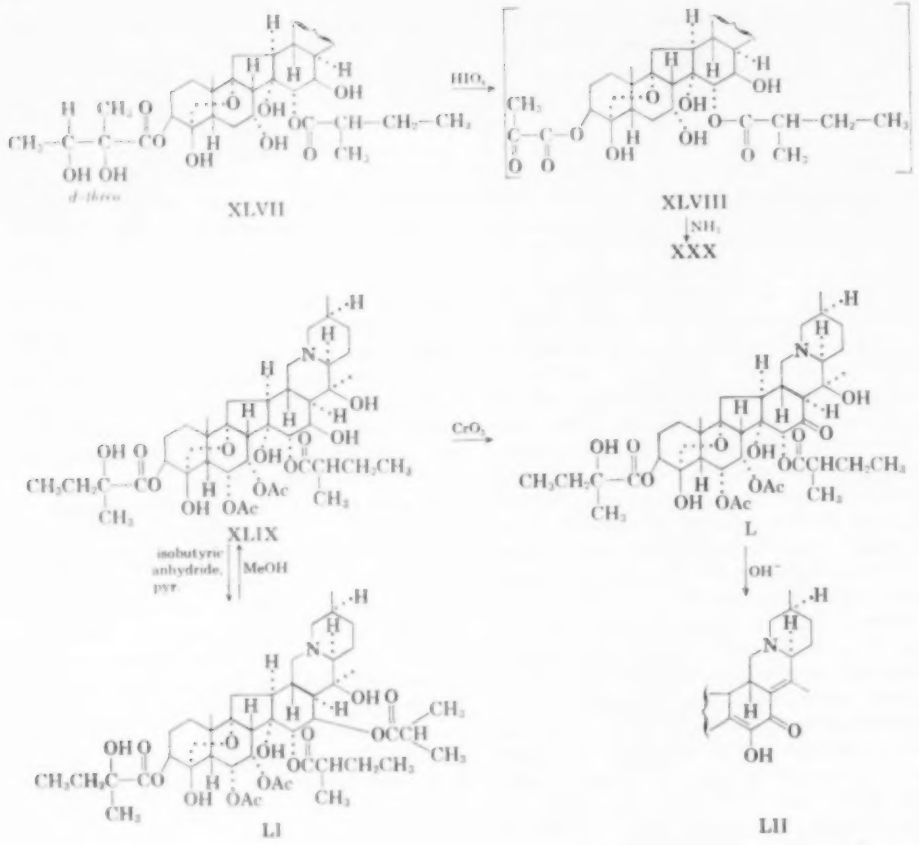


germidine is germine 3-acetate 15-(*l*)-2′-methylbutyrate (XL), and neogermidine is germine 7-acetate 15-(*l*)-2′-methylbutyrate (XLI).

Germanitrine (XLIV) was shown to be hydrolyzed with alkali to germine (XXIX), and one mole equivalent each of acetic, tiglic, and (*l*)-2-methylbutyric acids (35). Methanolysis of germanitrine resulted in loss of the acetate grouping with conversion to the diester germanidine. The latter compound yielded one mole equivalent of tiglic acid and one mole equivalent of (*l*)-2-methylbutyric acid on alkaline hydrolysis (35). Tentative assignment of the acetate group of germanitrine to C_7 of the germine nucleus was made on the basis of its reactivity toward methanolysis and its characteristically high contribution to the molecular rotation of the triester (33). The locations and natures of the remaining ester functions were demonstrated by partial synthesis of germanidine and germanitrine starting with germine. Partial synthesis of germine 3-tiglate 15-(*l*)-2′-methylbutyrate and of germine 3-(*l*)-2′-methylbutyrate 15-tiglate yielded diesters which differed significantly from germanidine (36). On the assumption that the tiglic acid formed upon hydrolysis might have arisen by isomerization prior to or during hydrolysis, a partial synthesis of germine 3-angelate 15-(*l*)-2′-methylbutyrate was next undertaken. Germine 15-(*l*)-2′-methylbutyrate (XXX) was treated with a limited amount of 3-bromoangeloyl chloride to yield XLV. The latter diester, on hydrogenolysis, afforded germine 3-angelate 15-(*l*)-2′-methylbutyrate (XLVI). The diester was stable to periodic acid, an indication that no free 1,2-glycol system was present. This fact limited the possible sites of attachment of the angelate residue to C_3 or C_4. The diester consumed two oxygen equivalents of chromic acid, which indicated that only two secondary hydroxyl groups were present and that the angelate residue was therefore affixed at C_3. The synthetic diester was identical with germanidine. Acetylation of XLVI with a limited amount of acetic anhydride yielded the triester germine 3-angelate 7-acetate 15-(*l*)-2′-methylbutyrate (XLIV). Evidence for the attachment of the acetate group at C_7 was obtained as follows. A chromic acid titration of the triester showed the presence of only one secondary hydroxyl group and C_4 was therefore excluded as a site for attachment of the acetate ester. Furthermore, the oxidation product, on alkaline treatment afforded a diosphenol with spectral properties identical with those of XXXIV. The synthetic triester was identical with germanitrine.

Germbudine (XLVII) was characterized by alkaline hydrolysis to germine, one mole equivalent of (*l*)-2-methylbutyric acid, and one mole equivalent of (*d*)-*threo*-2,3-dihydroxy-2-methylbutyric acid (39). On the basis of similarity of physical constants, the possibility that germbudine could be a diastereoisomer of neogermbudine was formulated as a working hypothesis for the elucidation of its structure. Germbudine, in agreement with the postulation of a C_3,C_{15} diester and the consequent presence of only one free glycol group (that of the dihydroxy acid) consumed 0.85 mole equivalent of periodic acid. The periodic acid titer did not distinguish between the C_3,C_{15} and the C_4,C_{15} diester structures. Evidence for the C_3,C_{15} structure (strongly favored on biogenetic grounds) was obtained by chromic acid titration of the diester. Germbu-

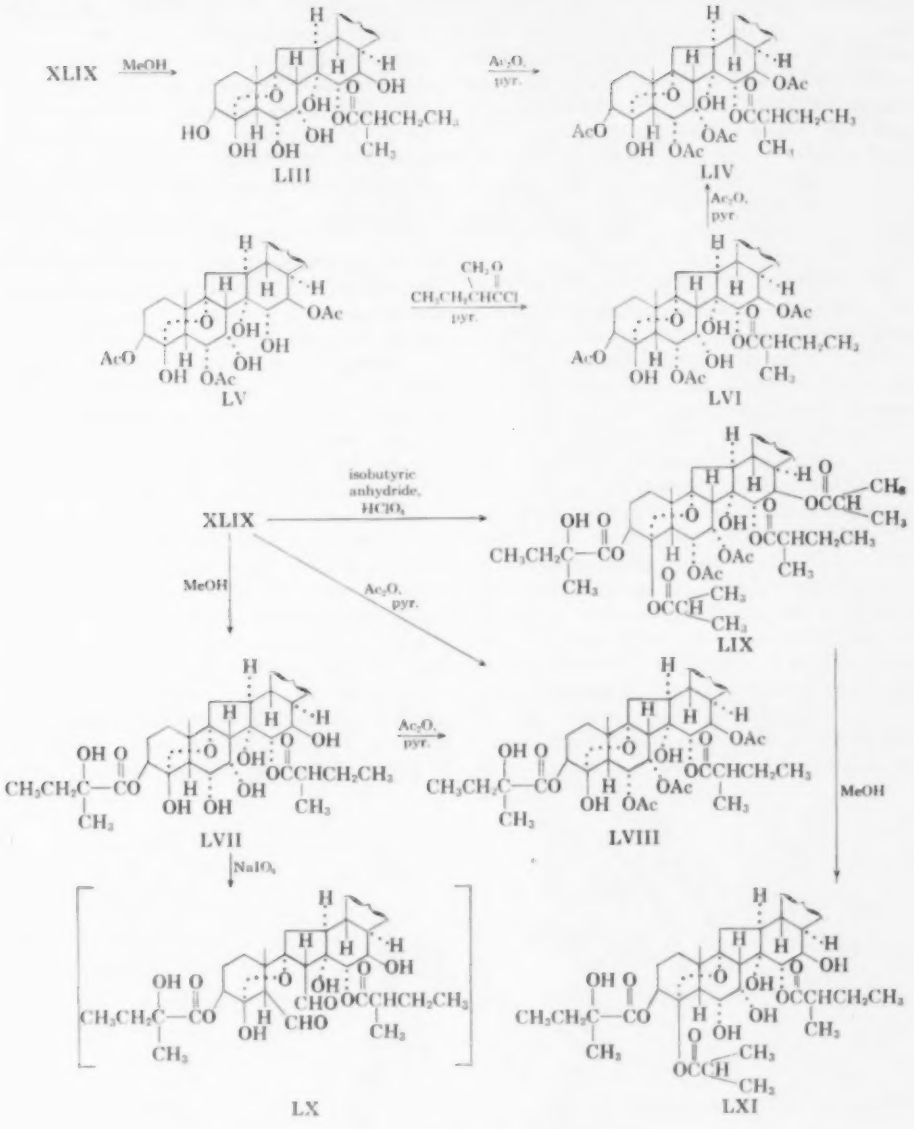




dine demonstrated a chromic acid consumption which paralleled that of neogermbudine and indicated the presence of three secondary hydroxyl groups (40). The specific location of each acyl group was determined by the selective removal of the (*d*)-*threo*-2,3-dihydroxy-2-methylbutyryl moiety. Oxidation of germbudine with periodic acid followed by treatment with aqueous ammonia yielded germine 15-(*l*)-2′-methylbutyrate. Hence, germbudine is germine 3-(*d*)-*threo*-2′,3′-dihydroxy-2′-methylbutyrate 15-(*l*)-2′-methylbutyrate (XLVII).

Protoveratrine A (XLIX) was cleaved by alkaline hydrolysis to protoverine (16) two mole equivalents of acetic acid, one of (*l*)-2-methylbutyric acid and one of (*d*)-2-hydroxy-2-methylbutyric acid (29, 30, 41, 42). Protoveratrine A consumed 0.9 oxygen equivalent of chromic acid, an indication that the C_4-hydroxyl group is not acetylated. The oxidation product, protoveratrone A (L) on alkaline hydrolysis afforded an amorphous diosphenol with spectral properties identical with those of the diosphenol (partial structure LII) obtained from alkaline hydrolysis of 16-dehydroprotoverine 3,4,6,7,15-pentaacetate (16). The latter experiment demonstrated that the C_{16}-hydroxyl group is not acylated in protoveratrine A. In keeping with only a single secondary hydroxyl group, protoveratrine A readily formed a monoacetate (LVIII) and a monoisobutyrate (LI). The fact that protoveratrine A could be obtained from methanolysis of the latter compound was advanced as supporting evidence for a free C_{16}-hydroxyl group in the naturally occurring tetraester (*cf.* ref. 16 for the facile methanolysis of C_{18} esters).

Vigorous methanolysis of protoveratrine A afforded a protoverine mono-(*l*)-2′-methylbutyrate (LIII). This compound consumed 1.9 mole equivalents of sodium periodate, an indication that the (*l*)-2-methylbutyrate residue was attached to the C_{18}-hydroxyl group. This was confirmed by acetylation to a tetraacetate shown to be protoverine 15-(*l*)-2′-methylbutyrate 3,6,7,-

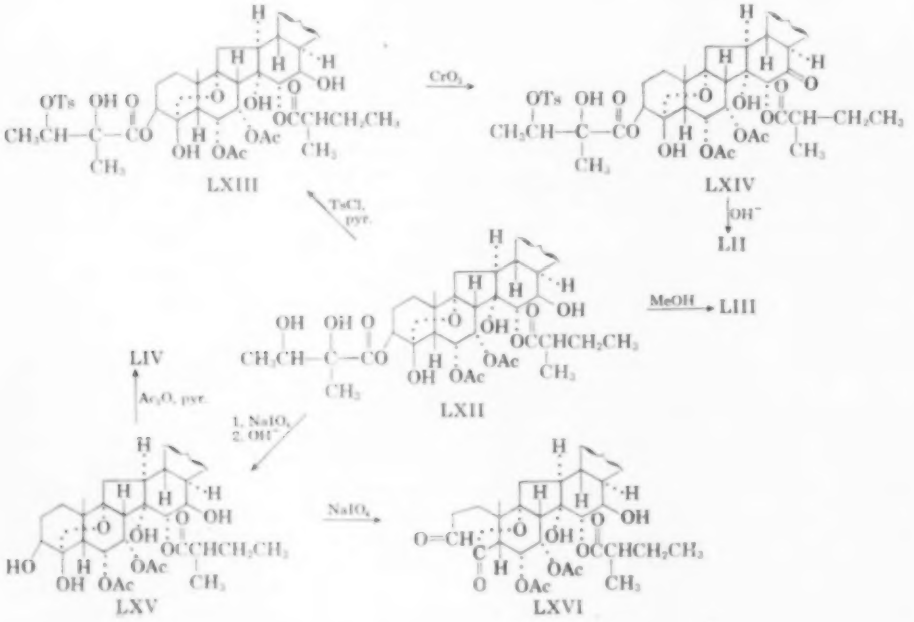


16-tetraacetate (LIV) by the sequence LV→LVI →LIV. A diester of protoverine, resulting from loss of both acetate groups, was also isolated from the methanolysis of protoveratrine A. Acetylation of this protoverine 15-(*l*)-2′-methylbutyrate (*d*)-2′-hydroxy-2′-methylbutyrate afforded protoveratrine A acetate (LVIII). The diester consumed 0.9 mole equivalent of sodium periodate and the spectrum of the amorphous oxidation product did not show the absorption characteristic of the γ-lactone formed by periodate cleavage of ring A of protoverine derivatives. The protoverine 3-(*d*)-2′-hydroxy-2′-methylbutyrate 15-(*l*)-2′-methylbutyrate (LVIII) assignment was supported by periodate-cyanometric titration data. Confirmation of the position of the 2-hydroxy-2-methylbutyric acid came from the following sequence. Protoveratrine A was converted to protoveratrine A 4,16-diisobutyrate (LIX), which on methanolysis (of the labile 16-isobutyrate and acetates) afforded protoverine 4-isobutyrate 15-(*l*)-2′-

methylbutyrate 3-(*d*)-2′-hydroxy-2′-methylbutyrate (LXI). If the hydroxybutyric acid were attached to either C_6 or C_7, the triester would be stable toward sodium periodate. In fact, the triester consumed 1.1 mole equivalents of sodium periodiate, thereby demonstrating that the hydroxybutyric acid was attached to the C_3-hydroxyl group. The foregoing facts proved that protoveratrine A is protoverine 3-(*d*)-2′-hydroxy-2′-methylbutyrate 6,7-diacetate 15-(*l*)-2′-methylbutyrate (XLIX).

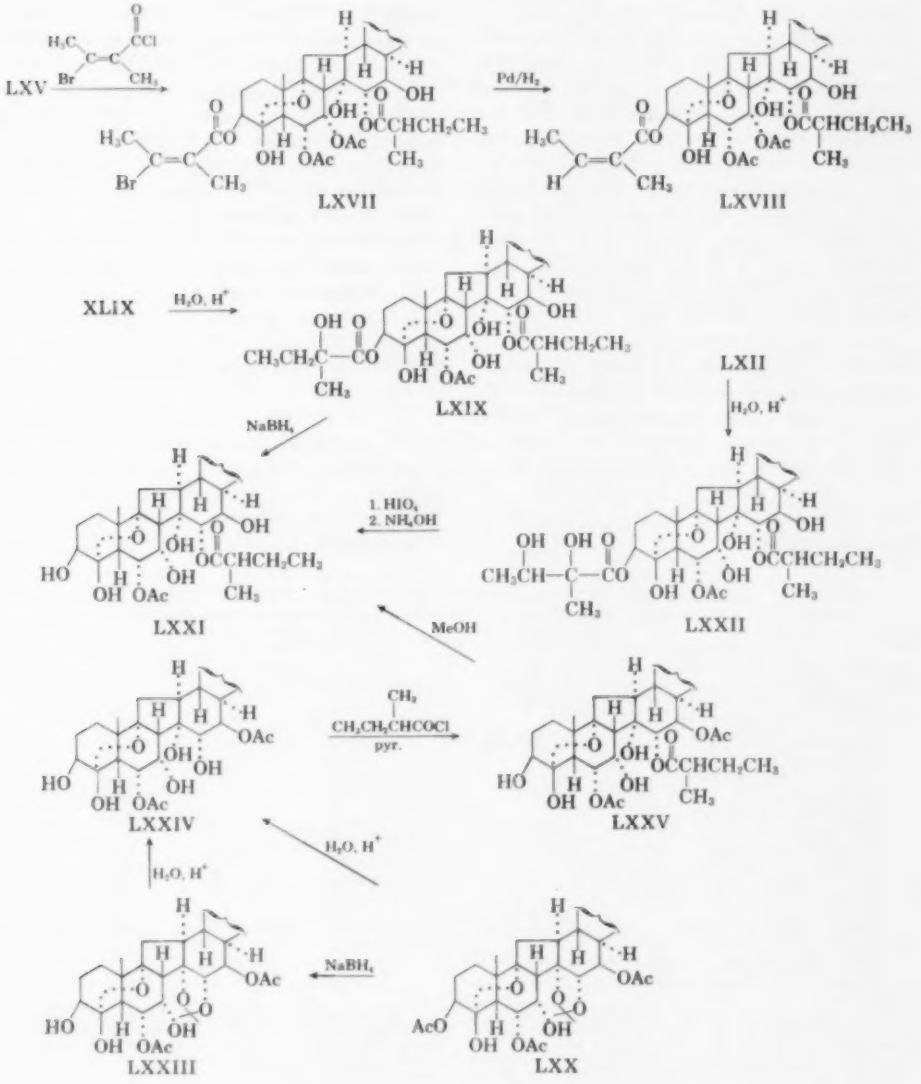
Protoveratrine B (LXII) hydrolysis gave protoverine, two molecular equivalents of acetic acid, one of (*l*)-2-methylbutyric acid, and one of (*d*)-*threo*-2,3-dihydroxy-2-methylbutyric acid (29, 30, 41, 42). Chromic acid titration of protoveratrine B showed the presence of two secondary hydroxyl groups, as indication that the C_4-hydroxyl group is not acylated. Protoveratrine B readily formed a monotosylate (LXIII) which was stable toward sodium periodate but consumed 1.1 oxygen equivalent of chromic acid. The oxidation product, dehydroprotoveratrine B monotosylate (LXIV), on alkaline hydrolysis afforded a diosphenol with spectral properties identical with those of the diosphenol from protoveratrine A. The latter facts demonstrated that in protoveratrine B the C_{16}-hydroxyl group is not acylated. Vigorous methanolysis of protoveratrine B afforded a protoverine mono-(*l*)-2′-methylbutyrate which was found to be identical with protoverine 15-(*l*)-2′methylbutyrate (LIII) previously isolated from the methanolysis of protoveratrine A. Oxidation of protoveratrine B with sodium periodate followed by dilute ammonia treatment led to the formation of a protoverine 15-(*l*)-2′-methylbutyrate diacetate (LXV). Acetylation of this compound gave protoverine 15-(*l*)-2′-methylbutyrate 3,6,7,-16-tetraacetate (LIV). On sodium periodate oxidation, the protoverine 15-(*l*)-2′-methylbutyrate diacetate consumed 1.0 mole equivalent. The oxidation product LXVI showed infrared absorption characteristic of the aldehydo-γ-lactone produced by periodate cleavage in ring A of protoverine derivatives. Hence protoveratrine B is protoverine 3-(*d*)-*threo*-2′,3′-dihydroxy-2′-methylbutyrate 6,7-diacetate 15-(*l*)-2′-methylbutyrate (LXII).

Escholerine (LXVIII) was characterized by hydrolysis to protoverine, two mole equivalents of acetic acid, one mole equivalent of (*l*)-2-methylbutyric acid and one mole equivalent of angelic acid (44). On biogenetic grounds, it was expected that the (*l*)-2-methylbutyrate and acetate residues might occupy the same positions as in protoveratrine A (XLIX) and protoveratrine B (LXII), and that the angelate residue might be attached at C_3. The structure was demonstrated by synthesis of protoverine 3-angelate 6,7-diacetate 15-(*l*)-2′-methylbutyrate. Treatment of triester LXV with a limited amount of 3-



bromoangeloyl chloride yielded protoverine 3-(3′-bromoangelate) 6,7-diacetate 15-(*l*)-2′-methylbutyrate (LXVII). The latter compound, on hydrogenation, afforded a product (LXVIII) identical with escholerine. The angelic acid was shown to be attached to C_3 in the following manner. A chromic acid titration of the tetraester showed the presence of only one secondary hydroxyl group and C_4 was thereby excluded as a site for attachment of the angelic acid ester. Furthermore, the oxidation product, on treatment with alkali, afforded diosphenol LII. Therefore escholerine is protoverine 3-angelate 6,7-diacetate 15-(*l*)-2′-methylbutyrate (LXVIII) (45).

Desacetylprotoveratrine A (LXIX) and desacetylprotoveratrine B (LXXII) were first characterized by hydrolysis to protoverine and the three respective acid moieties present in each triester (30, 39, 47). The nature of each triester was subsequently confirmed by degradation of protoveratrine A to desacetylprotoveratrine A and of protoveratrine B to desacetylprotoveratrine B by methanolysis (30, 47) and by acid hydrolysis (46). Treatment of desacetyl-



protoveratrine B with periodic acid followed by exposure of the crude oxidation mixture to dilute ammonium hydroxide selectively removed the dihydroxymethylbutyryl residue and yielded a protoverine (*l*)-2′-methylbutyrate acetate diester (LXXI). Sodium borohydride reduction of desacetyl protoveratrine A afforded the same diester. The structure of diester LXXI was proved by the following sequence. Protoverine 14,15-acetonide 3,6,16-triacetate (LXX) was converted to protoverine 6,16-diacetate (LXXIV) by prolonged mineral acid hydrolysis or, alternatively, by sodium borohydride reduction to LXXIII followed by hydrolysis to LXXIV. Treatment of LXXIV with a limited amount of (*l*)-2-methylbutyryl chloride gave protoverine 15-(*l*)-2′-methylbutyrate 6,16-diacetate (LXXV). The product obtained upon sodium periodate oxidation of LXXV (consumption: 1.1 mole equivalents) showed infrared absorption characteristic of the aldehydo-γ-lactone absorption. Hence desacetylprotoveratrine A is protoverine 3-(*d*)-2′-hydroxy-2′-methylbutyrate 6-acetate 15-(*l*)-2′methylbutyrate (LXIX) and desacetylprotoveratrine B is protoverine 3-(*d*)-*threo*-2′,3′-dihydroxy-2′-methylbutyrate 6-acetate 15-(*l*)-2′-methylbutyrate (LXXII) (47).

The relative hypotensive activity of those veratrum ester alkaloids for which comparative data are available is listed in Table II. The pharmacological data indicates that the number, the nature, and the positions of the esterifying acids are of importance in determining the degree of hypotensive activity. The structure-activity relationships which are apparent from the data in Table II follow:

(*a*) In the germine series, the 3,7,15-triesters are the most active compounds. Removal of the C_7-acetate group from a germine 3,7,15-triester is accompanied by a diminution in hypotensive potency (compare germitetrine (XXVIII) *vs.* desacetylgermitetrine (XXXI) [relative Myers activity, 1.8 (30)]; neogermitrine (XXXIX) *vs.* germidine (XL); germitrine (XXXV) *vs.* germerine (XXXVI); germanitrine (XLIV) *vs.* germanidine (XLVI) [relative "Maison" activity, 1.3 (35)].

(*b*) Among the germine 3,7,15-triesters, variations in the nature of the ester group affixed at C_3 are not accompanied by particularly marked changes in hypotensive potency [compare germitetrine (XXVIII) *vs.* neogermitrine (XXXIX) *vs.* germanitrine (XLIV)].

(*c*) Among the germine diesters, the 3,15-derivatives show a relatively high order of activity [compare germerine (XXXVI), germidine (XL), germbudine (XLVII), and neogermbudine (XXXII) *vs.* neogermidine (XLI)].

(*d*) In the protoverine series, the 3,6,7,15-tetraesters are the most active compounds. Removal of the C_7-acetate group from a protoverine 3,6,7,15-tetraester is accompanied by a diminution in hypotensive potency [compare protoveratrine A (XLIX) *vs.* desacetylprotoveratrine A (LXIX); protoveratrine B (LXII) *vs.* desacetylprotoveratrine B (LXXII)].

(*e*) Among the protoverine 3,6,7,15-tetraesters, variations in the nature of the ester group affixed at C_3 are not accompanied by particularly marked changes in hypotensive potency [compare protoveratrine A (XLIX) *vs.* protoveratrine B (LXII) *vs.* escholerine (LXVIII)].

Table II.—Relative Hypotensive Activity of Certain Veratrum Ester Alkaloids

Alkaloid	Relative Hypotensive Activity: Maison[a]	Myers[b]	References
Veratroylzygadenine (XII)	0.9	...	(35, 51)
Cevadine (XXII)	0.18	...	(52)
Veratridine (XXVI)	0.5	...	(52)
Germitetrine (XXVIII)	...	2.1	(30)
Germitrine (XXXV)	11.0	...	(52)
Neogermitrine (XXXIX)	8.7	...	(52)
Germanitrine (XLIV)	8.3	...	(35)
Germinitrine	2.4	...	(35)
Germerine (XXXVI)	5.3	...	(52)
Germidine (XL)	2.4	...	(52)
Neogermidine (XLI)	...	0.1	(39)
Germbudine (XLVII)	...	0.8	(39)
Neogermbudine (XXXII)	...	1.0	(30)
Protoveratrine A (XLIX)	5.4	...	(41, 53)
Protoveratrine B (LXII)	4.0	1.5	(39, 41, 53)
Escholerine (LXVIII)	3.3	...	(44)
Desacetylprotoveratrine A (LXIX)	...	0.4	(30)
Desacetylprotoveratrine B (LXXII)	1.0	0.4	(39, 47)

[a] Activity relative to a mixed alkaloidal ester preparation (Veriloid, activity = 1.0) which produced a 30% fall in mean arterial pressure when administered at a dose of 1 mcg./Kg./min. by intravenous injection of ten minutes duration in anesthetized dogs (35, 41, 44, 47, 51, 52, 53).

[b] Activity relative to a mixed alkaloidal ester preparation from *Veratrum viride* (Deravine, activity = 1.0) which produced a 30% fall in mean arterial pressure of the anesthetized dog at a dose of 2 mcg./Kg. administered intravenously over a ten-minute period (30, 39).

Pharmacological evaluation of series of synthetic germine esters (54, 55) and of synthetic protoverine esters (55–57) have recently yielded results which supplement and extend the structure-activity generalizations revealed in the studies of the naturally occurring hypotensive ester alkaloids. Studies on the emetic properties of the hypotensively active ester derivatives are currently under way, and new relationships between structure and emetic activity in this series may be forthcoming soon from the latter investigation. It is hoped that such data, coupled with the aforementioned generalizations between structure and hypotensive potency, might constitute excellent guides for the synthesis of useful hypotensive agents (58).

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Investigation of Some Factors Influencing Percutaneous Absorption

By DALE E. WURSTER and SHERMAN F. KRAMER†

A method has been developed for determining relative percutaneous absorption rates of liquid chemical agents in humans. Experimental data indicated that moisture conditioning of the skin surface accelerated the absorption rate of three salicylate esters, the greatest effect being observed with the ester having the lowest oil/water distribution coefficient. First-order urinary elimination rates following percutaneous absorption were established for all the compounds tested. Defatting of the skin surface with ether decreased the absorption rate of methyl salicylate. A linear relationship between the steady state elimination levels and the surface area of the skin exposed to ethyl salicylate indicated that the rate limiting step in the experimental system was the diffusion of the salicylate ester through the skin.

THE OBJECT in most previous percutaneous absorption studies was mainly to show whether absorption into or through the skin occurred and thus were only qualitative in nature. In some recent studies attempts have been made not only to obtain quantitative data but to ascertain the pathway of penetration and to determine the chemical and physical properties which influence percutaneous absorption (1–8).

In general, *in vivo* studies to date have met with only limited success in producing quantitative and meaningful data. It is also recognized that *in vitro* studies are limited by the possibility that excised skin may vary greatly from that on the living animal. Also, it is doubtful, because of reasons too numerous to mention here, whether data obtained with experimental animals can be used to explain the phenomenon of percutaneous absorption in humans (9, 10).

The current study was therefore initiated in an attempt to obtain quantitative data and to elucidate some of the physicochemical factors which affect percutaneous absorption in humans.

METHOD OF STUDY

It was assumed in this study that percutaneous absorption involves a diffusional process: that is, the spontaneous movement of the test substance from an area of high concentration on the external skin surface to an area of lower concentration in the tissue fluids. Also, it was recognized that when a drug is incorporated into an ointment base and the ointment then applied to the skin, the process must be considered in at least two distinct phases: (*a*) the diffusion or leaching of the drug from the base to the skin surface, and (*b*) the passage of the drug into and through the various skin substances and structures. Thus, to avoid the complication of a base or solvent, it was necessary to work with the pure penetrant on the skin surface.

Of the many methods that have been used to detect percutaneous absorption in the live animal (11), indications were that a procedure involving the direct analysis of urine would provide reliable proof of absorption as well as offer the least discomfort to the experimental subject.

Three liquid salicylate compounds (ethyl salicylate, methyl salicylate, and ethyleneglycol salicylate), because of their distribution coefficients (olive oil/water), were selected for the study. Previous work (12–15) indicated that 75 to 90% of the total salicylate taken into the body could be accounted for by urine analysis. The total recoverable salicylate in the urine was determined following intravenous doses of sodium salicylate in human test subjects.

It was expected that a steady or constant rate of elimination of salicylate in the urine would be obtained if the exposed skin area and the penetrant concentration on the skin surface remained constant. This state would not be realized until an equilibrium was established between the processes of absorption, distribution, metabolism, and excretion of the salicylate. If the analytical method produced consistent results with the formed metabolites, the metabolic fate of the salicylate need not complicate the proposed scheme so long as the per cent of salicylate unaccounted for did not vary significantly.

The increased absorption of chemical substances through the skin under conditions of a high hydration state of the skin has been suspected by several workers (15–19). This effect was studied by obtaining data involving two extremes of moisture conditioning at the skin surface.

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The effect of defatting the skin surface with solvents has been reported to both hinder (20–22) and aid (16, 23) the penetration of various chemical substances. The absorption of methyl salicylate following the defatting of the skin surface with ether was also studied.

EXPERIMENTAL

Procedure.—The analytical procedure employed for determining total urine salicylate was a modification of the Brown and Scott (24) method. The method consisted of the acid hydrolysis of the salicylate conjugates with subsequent steam distillation of the free salicylic acid. A mixture containing 250 ml. of the urine sample and 20 ml. of 85% phosphoric acid was concentrated until the temperature reached 120°. Steam distillation was then initiated and the temperature increased to 140°. Continuous steam distillation was carried out until the distillate was shown to be devoid of salicylic acid when tested with ferric nitrate test solution (5% ferric nitrate in 0.1 *N* nitric acid).

To a 50-ml. sample of the distillate ferric nitrate test solution (1 ml.) was added to develop the characteristic color of the iron-salicylate complex. The absorbance of this solution was determined at 526 mμ using a Cary recording spectrophotometer equipped with 10-cm. silica cells. A solution containing an equivalent amount of the test solution in distilled water was employed as the blank.

Many of the distillates possessed a slight yellow color which gave a slight interference at 526 mμ. Correction of this error was accomplished by comparing a sample of the plain distillate with distilled water. The difference was used as a correction factor in evaluating the absorbance of the solution containing the distillate and ferric nitrate test solution.

Blank Urine Determinations (Error Estimation).—Various phenolic substances have been shown to be present in the urine (25). To determine what effect these might have, the following test was performed. Prior to exposure to salicylate, urine samples were taken from each of the test subjects every two hours for a sixteen-hour period. The samples were assayed according to the procedure for total salicylate. Absorbances were then used to calculate the equivalent amount of salicylic acid.

Recovery of Salicylate in Urine after Intravenous Injection of Sodium Salicylate.—Each experimental subject received a 250-mg. intravenous dose of sodium salicylate (equivalent to 215.7 mg. of salicylic acid). Urine samples were taken from each individual every two hours for a period of twenty-four hours. The urine samples were analyzed for total salicylate in the manner described above. From the analytical results, the per cent total recovery was calculated and the first-order excretion constants determined.

Long Term Exposure of the Skin to Penetrant under Hydrous and Anhydrous Conditions.—In order to maintain a fixed boundary around a measured area of skin surface and to expose the skin to an excess of the penetrant for prolonged periods of time, a plastic cell (1 × 5 × 10 cm.) which could be affixed to the forearm was designed (Fig. 1). This cell was molded over a plaster form of a specific area of the arm of each individual subject. The

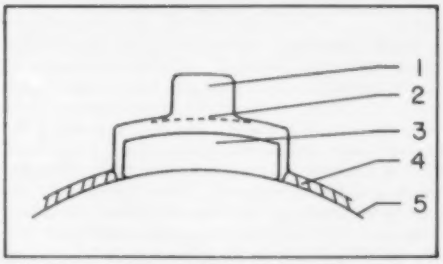

Fig. 1.—Cross section of absorption cell. 1, Desiccant compartment; 2, filter paper floor; 3, sponge material; 4, layers of tape and cement by which shoulder of the plastic cell was fixed to the skin surface; 5, skin surface.

desired skin area was then defined and the border painted with Duco cement diluted with acetone. Over this border, strips of adhesive tape were placed and painted with a layer of the cement. The shoulder of the plastic cell was then cemented to the tape. Inside the cell, prior to cementing it to the forearm, a 5 × 10-cm. sponge was placed. At the start of the experiment the sponge was filled with 6 ml. of the penetrant and 3 ml. of distilled water. During the exposure time urine samples were taken every two hours. At the end of sixteen hours the cell was removed and the area cleansed with warm water and soap. In some cases urine collection continued after removal of the cell until the urine tested negative for salicylate. The total excreted salicylate (calculated as the salicylate ester) was plotted against time and the first-order excretion constants determined.

To create an anhydrous condition the desiccant compartment of the cell was filled with magnesium perchlorate. This compartment contained a filter paper floor which was cemented in place. This kept the conditions within the cell at the low extreme of moisture content. The sponge was again filled with 6 ml. of the penetrant but no water. The time of exposure and urine sampling were the same as described for the hydrous experiments.

Correlation of Exposed Skin Area to Absorbance Rate.—Using the described plastic cells and analytical method, varying areas of skin surface (25 to 100 cm.^{2}) of the forearm were exposed to ethyl salicylate for a period of sixteen hours. The rates of urinary salicylate excretion (calculated as ethyl salicylate) were correlated with the area exposed to the salicylate.

Methyl Salicylate Absorption Through Ether Defatted and Nondefatted Skin.—For the defatting procedure, the arms of the test subjects were immersed in ethyl ether for a period of one minute. An excess of methyl salicylate was maintained on an area of 100 cm.^{2} on each forearm for a two-hour period. The area was then cleansed with water and soap. Urine samples were collected every two hours until they tested negative for salicylate. The same procedure was carried out on nondefatted skin surfaces of the forearms. The total recovered salicylate for the two procedures was then compared graphically and the first-order excretion constants determined.

RESULTS AND DISCUSSION

Blank Urine Determination (Error Estimation).—Alpen (26) cautioned that one of the difficulties of the iron-complex method for salicylic acid determination in urine is that this method produces color with any phenolic group. Figure 2 represents the average error possibility caused by such miscellaneous phenolic substances in the urine distillates for a sixteen-hour period. Since the conditions used in determining this variation follow the conditions used in the actual experiments, this figure represents the magnitude of error expected from this source. It was considered relatively insignificant compared to urinary salicylate values found in the absorption experiments.

Fig. 2.—Error produced by contaminants in urine distillates.

Recovery of Salicylate in Urine Following I. V. Sodium Salicylate.—The total recovered urine salicylate varied between 83.8 and 94.5% (av. 89.9%) following a 250-mg. intravenous dose (equivalent to 215.7 mg. salicylic acid) of a commercial sodium salicylate preparation. As shown in Fig. 3 a straight line relationship was obtained when the logarithm of the salicylate concentration in the two-hour urine samples was plotted against time. The data thus adhere to the following first-order rate expression:

$$-dS/dt = kS$$

where S is the salicylate present in the body, t is the time in hours, and k is the rate constant.

On integration,

$$k = \frac{2.3\ (\log S_1 - \log S_2)}{t} = \frac{2.3\ (\log 20 - \log 2)}{8}$$

$$k = 0.29\ \text{hours}^{-1}$$

and

$$t_{1/2} = \frac{0.693}{k} = \frac{0.693}{0.29} = 2.4\ \text{hours}$$

Other workers have also reported the first-order elimination of salicylate (27).

Long Term Exposure of the Skin to Penetrant under Hydrous and Anhydrous Conditions.—Figures 4, 5, and 6 show graphically the summarized data for the prolonged experiments under hydrous and anhydrous conditions for the three salicylate esters. From these plots it can be observed that the rate ultimately becomes constant and thus follows the zero-order expression:

$$dS/dt = k$$

where, again, S is the salicylate present in the body, t is the time in hours and k is the rate constant. That is, the rate of excretion becomes a constant k

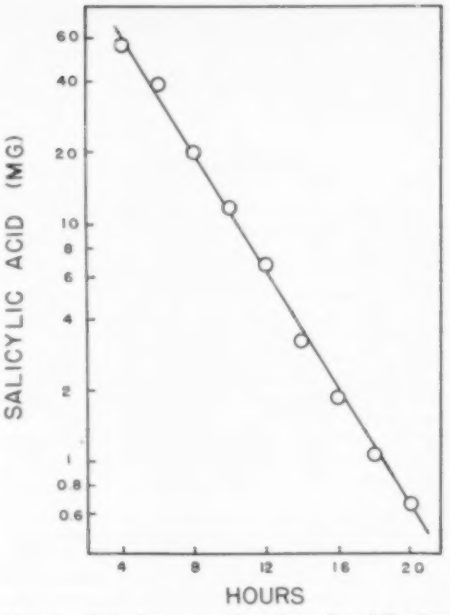

Fig. 3.—Salicylate excretion in urine following intravenous sodium salicylate.

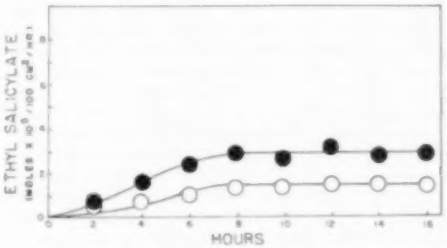

Fig. 4.—Urinary excretion data showing the influence of moisture on the percutaneous absorption rate of ethyl salicylate. ● Hydrous system rate, 2.9 (plateau level); ○ anhydrous system rate, 1.5 (plateau level).

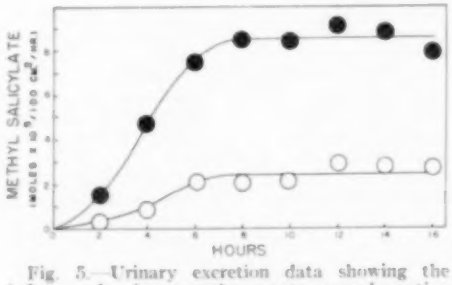

Fig. 5.—Urinary excretion data showing the influence of moisture on the percutaneous absorption rate of methyl salicylate. ● Hydrous system rate, 8.6 (plateau level); ○ anhydrous system rate, 2.7 (plateau level).

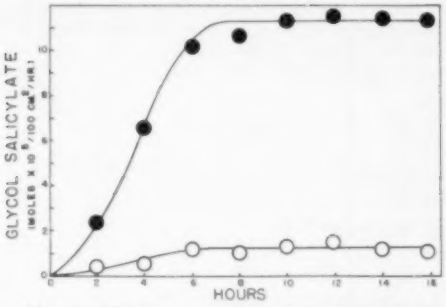

Fig. 6.—Urinary excretion data showing the influence of moisture on the percutaneous absorption rate of glycol salicylate. ● Hydrous system rate, 11.7 (plateau level); ○ anhydrous system rate, 1.3 (plateau level).

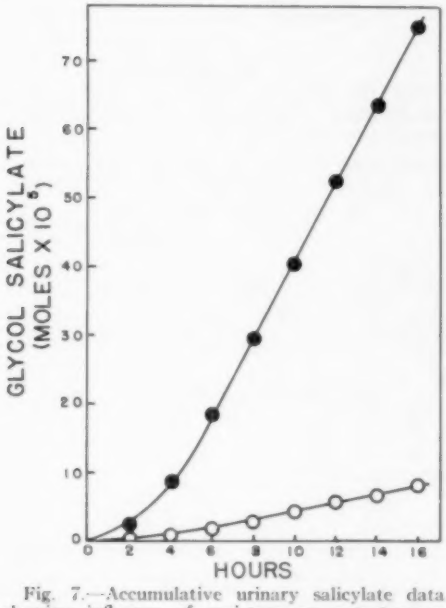

Fig. 7.—Accumulative urinary salicylate data showing influence of moisture on percutaneous absorption rate of glycol salicylate. ● Hydrous system rate, 11.7; ○ anhydrous system rate, 1.3.

as determined by the rate of diffusion through the skin. The value for salicylic acid found in the distillate was converted to milligrams of salicylate ester and plotted as such. No correction was made to increase this value to the probable actual amount of salicylate diffusing through the skin.

The data can also be shown as accumulative plots of which Fig. 7 is a typical example. After about the sixth hour, in all cases, the total salicylate value increased in direct proportion to time. That is, the excretion rate is at a steady state. The slope of this line is the excretion rate of the salicylate (calculated as the salicylate ester).

The data presented in each of the preceding graphs (Figs. 4–7) represent the average values obtained from ten experiments with the same salicylate in the case of the hydrous skin condition and eight experiments in the anhydrous condition. Graphs constructed from data obtained in the individual experiments were, of course, similar to those obtained with the average values.

Table I is a summary of the experimental excretion rates (relative percutaneous absorption rates) and other physical constants of the test penetrants. In No. 3 the ratio of the rate found for the hydrous system to the rate found for the anhydrous system is given. This ratio expresses the increase in rate caused by the presence of excess moisture at the skin surface. It is evident that this ratio decreases with the decreasing water solubility of the salicylate (No. 4) or decreases with an increase in the oil/water distribution coefficient (Nos. 5 and 6). Thus, it can be seen that the more water-soluble compound is aided to a greater degree by the presence of moisture than those of lesser solubility. This trend is not directly proportional to the increasing water solubility or to the decreasing distribution coefficient. This probably should not be expected since other physical phenomena such as viscosity, molecular size, intermolecular bonding, adsorption, etc., can be expected to play roles of varying significance for each compound. For example, ethyleneglycol salicylate has a limiting solubility in oil (16% in olive oil) while the ethyl and methyl esters are completely miscible. Upon encountering a lipid barrier in the skin, this lower solubility would be expected to hinder the passage of the glycol derivative much more than either the ethyl or methyl salicylates. Supporting evidence on the effect of increased moisture can be found in the recent literature for these observations. Dutkiewicz (19) reported that cutaneous absorption of aniline was increased by 50% when the air humidity was raised by 40%. Piotrowski (17) at about the same time (1957) reported that the amount of aniline absorbed increased with skin temperature and skin moisture content. Meigs and co-workers (28), in a study of workers assigned to the production of benzidine, dichloro-

Table I.—Experimental Excretion Rates and Other Physical Constants of the Test Penetrants

No.		Glycol Salicylate	Methyl Salicylate	Ethyl Salicylate
1	Hydrous system rate (moles/100 cm.²/hr.) calculated as the salicylate ester	11.7	8.6	2.9
2	Anhydrous system rate (moles/100 cm.²/hr.) calculated as the salicylate ester	1.3	2.7	1.5
3	Rate hydrous system / Rate anhydrous system	9.0	3.2	2.0
4	Per cent water solubility	1.27	0.08	0.03
5	Distribution coefficient (olive oil/water)	7.7	343	1,170
6	Relative distribution coefficient (glycol salicylate 1)	1	45	152

benzidine, dianisidine, and *o*-tolidine, showed absorption by urine examination. They implicated the skin as the main portal of entry and further stated that high air temperature and high humidity accelerated the penetration of these substances.

It appears that in future studies of percutaneous absorption dealing with drug systems placed on the skin, consideration should be given to the hydration state of the skin under study in order to obtain valid comparisons of rates.

Excretion Rates.—It was noted throughout this study that the excretion rates after absorption was terminated were first order and appeared nearly identical for the compounds used or the experimental conditions imposed. The intravenous sodium salicylate indicated the same half-life and k value shown by the three salicylate esters applied to the skin. Figure 8 shows the first-order elimination rates. The k values are 0.29 ± 0.01 hours^{-1} and the $t^1/_2$ values are 2.2 ± 0.2 hours.

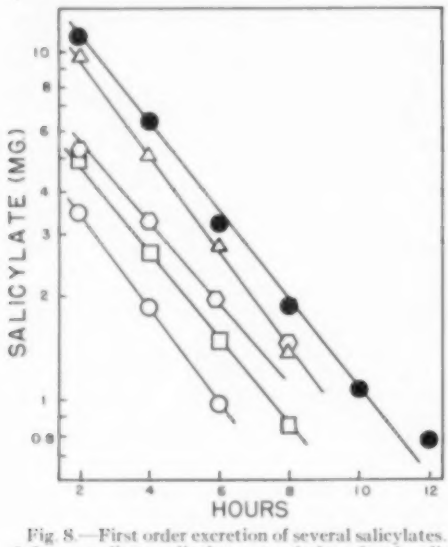

Fig. 8.—First order excretion of several salicylates. ● I. v. sodium salicylate, Δ ethyleneglycol salicylate, ◯ ethyl salicylate, □ methyl salicylate, ○ methyl salicylate (ether defatted skin).

Correlation of Exposed Skin Area to Absorption Rate.—If the excretion of salicylate in the urine is determined by the amount of the salicylate ester passing through the skin barrier per unit of time, then the excretion rate should be directly proportional to the area of skin surface through which the salicylate diffuses. Figure 9 is a plot of the steady state excretion levels established (calculated as mg. ethyl salicylate) *versus* total area exposed. Since a direct proportionality relationship does exist, this tends to support the assumption that the rate limiting step in this experimental system is the diffusion of the salicylate ester through the skin.

Methyl Salicylate Absorption Through Ether Defatted and Nondefatted Skin.—Following the defatting of the skin surface with ethyl ether a decrease of 27% was noted in the total salicylate absorption. Figure 10 is a representative accumulative plot of the methyl salicylate elimination. When the logarithm of urinary salicylate was plotted against time in this case a first-order elimination was again indicated. The k value was found to be 0.31 hours^{-1} and the $t^1/_2$ value 2.2 hours (see Fig. 8).

The difference in absorption noted may be due to the fact that the lipid pathway through which the penetrant normally diffuses has been disrupted or partially eliminated. It may also be that the ether extraction removes significant amounts of water or indirectly produces a partially dehydrated state by the extraction of lipid-soluble materials and other substances responsible for the water hold-

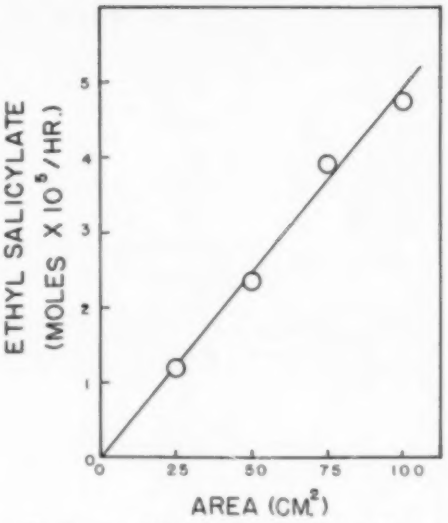

Fig. 9.—Correlation of salicylate treated skin area (ethyl salicylate, hydrous system) with salicylate urinary excretion rate.

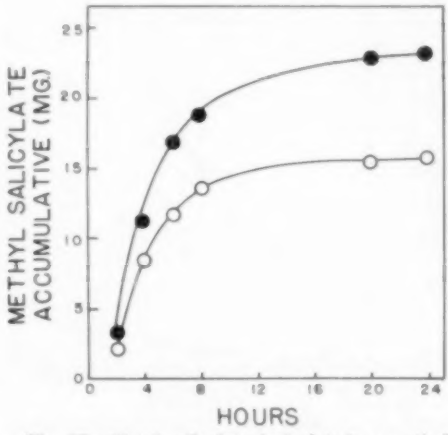

Fig. 10.—Total salicylate (calculated as methyl ester) recovered from urine. ● Nondefatted skin, ○ ether defatted skin.

ing capacity of the skin (29). The resulting dry condition possibly offers a more effective barrier to chemical penetration in much the same manner as noted in the hydrous and anhydrous experiments above.

SUMMARY

A method for studying the percutaneous absorption of liquids in humans has been developed and tested to the point where the accuracy and control of variables is sufficient to yield meaningful data which can be handled mathematically. The method depends upon the established rate of excretion of total salicylate in urine being a reliable estimate of the relative rate of penetration through the skin.

The use of hydrous and anhydrous cell systems demonstrated that a large increase in the absorption rate was caused by increased moisture conditioning of the skin.

The magnitude of the effect of moisture on percutaneous diffusional rates has been shown to be proportional to the oil/water distribution coefficient and the water solubility of the closely related test substances. The greatest increase of the penetration rate by moisture was found in the penetrant with the smallest oil/water distribution coefficient.

First-order elimination rates following percutaneous absorption were established for three salicylate compounds. Nearly identical k and $t_{1/2}$ values were found for all salicylates used in this study.

A linear relationship between the steady state salicylate excretion levels and the surface area of the skin exposed to ethyl salicylate was found. This indicates that the rate limiting step in the experimental system employed is the diffusion of the salicylate ester through the skin.

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Study of Anionic and Cationic Surfactants in a Hydrophilic Ointment Base I

The Screening Program

By KANU C. PATEL†, GILBERT S. BANKER, and H. GEORGE DeKAY

Sixty-two anionic and twenty-eight cationic surfactants were subjected to two screening procedures to determine which surfactants warranted a complete evaluation in the hydrophilic ointment formula. Following the preliminary screening, the remaining ten anionic and five cationic surfactants were evaluated for stability, spreadability, washability, consistency, and water loss in the hydrophilic ointment base. Both the type and concentration of the surfactant used affected the water-retention property of the respective bases. The ointment base prepared with a two per cent concentration of the anionic surfactant, sodium lauryl ether sulfate, produced the most satisfactory hydrophilic ointment vehicle based on pharmaceutical elegance and compatibility with representative topical drugs.

EMULSIFIED OINTMENT BASES have been recognized as nearly ideal vehicles for topical medication during the last two decades. Oil-in-water type emulsion bases have several advantages over and are preferred to water-in-oil emulsion ointment bases (1, 2). A representative example of an oil-in-water type emulsion base became official in the U. S. P. XIII under the title of hydrophilic ointment and has been used extensively since that time (3). The recent development of a wide variety of surfactants has stimulated many workers to evaluate them for use in the formulation of a better hydrophilic ointment. Nonionic surfactants have been rather extensively evaluated for their use in hydrophilic ointment. The U. S. P. XV replaced sodium lauryl sulfate in the official hydrophilic ointment and introduced a nonionic surfactant, polyoxyl 40 stearate (Myrj-52). The U. S. P. XVI however has reverted to the sodium lauryl sulfate formula. Barker and DeKay (4) showed that the hydrophilic ointment U. S. P. XIV in which an anionic surfactant, sodium lauryl sulfate, is used has a greater release of medication than the other ointment bases containing nonionic surfactants. It has been claimed that nonionic surfactants are less irritating than the anionic or cationic types (5); however, several ionic surfactants have received widespread use in pharmaceutical products. A few anionic and cationic surfactants have been studied in the formulation of a hydrophilic ointment base (6, 7). The newly developed anionic and cationic surfactants have not been studied extensively in pharmacy. Some recently developed lauryl ether sulfate anionic surfactants have been used in high concentrations in a surgical scrub soap formulation and in cream shampoos, without producing any appreciable irritating effect (8, 9).

The primary purpose of this investigation was to study specific pharmaceutical applications of anionic and cationic surfactants through an evaluation of the hydrophilic ointment bases produced. To reach this objective, (*a*), it was necessary to screen a large number of surfactants to provide reasonable assurance that the most desirable agent or agents of the group currently available was selected, and (*b*), careful consideration had to be given to the evaluation procedures to obtain a valid objective evaluation of the surfactants studied. The penetrometer has been reported to successfully measure ointment consistency (10, 11). Singiser and Beal (11) reported that the penetrometer was a good tool for measuring the consistency of ointment samples with penetration reading of less than four hundred.[1]

The tendency of emulsion bases to lose water by evaporation has always been a matter of concern. Several workers (12, 13) have reported different methods for the determination of water loss from emulsified ointment bases. Lesshafft and DeKay (10) studied the effect of nonionic surfactants on the water retention properties of ointment bases. A survey of the literature shows that anionic and cationic surfactants have not been studied for their water retention properties in any emulsion system.

EXPERIMENTAL

Sixty-two anionic and twenty-eight cationic surfactants were selected for use in this study on the basis of their chemical form, purity, potential sta-

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[1] The consistency is measured as the depth, in tenths of a millimeter, of the standard cone during a period of five seconds.

TABLE I.—ANIONIC AND CATIONIC SURFACTANTS AS EMULSIFYING AGENTS FOR HYDROPHILIC OINTMENT

Tradename[a]	Chemical Designation	First Screening Evaluation
	Anionic Surfactants	
Aerosol AY	Diamyl ester of sodium sulfosuccinic acid	Grainy[b]
Aerosol MA	Dihexyl ester of sodium sulfosuccinic acid	Grainy
Aerosol OS	Isopropyl naphthalene sodium sulfonate	Separation[c]
Aerosol OT	Dioctyl ester of sodium sulfosuccinic acid	Satisfactory[d]
Aerosol 18	Disodium N-octadecylsulfosuccinamate	Satisfactory
Aerosol 22	Tetrasodium N-(1,2-dicarboxyethyl)-N-octadecylsulfosuccinamate	Satisfactory
Alromine RA	Fatty alkyloamine condensate	Satisfactory
Artic syntex M	Sodium oleyl methyl taurate	Unsatisfactory[e]
Alkanol B	Sodium alkyl naphthalene sulfonate	Separation
Acto 630	Alkyl aryl sulfonate	Unsatisfactory
Antaron S-120	Sulfonated fatty acid	Grainy
Antaron S-291	Sulfonated fatty acid	Satisfactory
Antaron S-370	Sulfonated fatty acid	Separation
Alrosperse 11-P	Fatty amine condensate	Separation
Antaron K-430	Alkyl aryl polyethylene sulfate	Unsatisfactory
Alropon Conc.	Salt of secondary alcohol sulfate	Satisfactory
Agent TR	Dimyristyl sodium sulfosuccinamate	Grainy
Agent BPE	*p-tert*-Butylphenoxyethyl sodium sulfosuccinate	Satisfactory
Alipal CO-436	Ammonium salt of sulfate ester of an alkyl phenoxy polyoxyethylene ethanol	Grainy
Antarate K	Alkyl carboxylate	Satisfactory
Blendene	Terpene fatty acid salt complex	Unsatisfactory
Detergent G	Dodecylbenzene sodium sulfonate	Separation
Duponol WA paste	Technical lauryl sodium sulfate	Satisfactory
Duponol LS Flakes	Technical oleyl alcohol sulfate	Satisfactory
Duponol ST	Fatty alcohol triethanolamine	Satisfactory
Igepon AP-78	Oleic acid ester of sodium isothionate	Satisfactory
Igepon T-73	Sodium N-methyl N-oleyl taurate	Satisfactory
Lathanol LAL	Sodium lorol sulfoacetate	Satisfactory
Marasperse CB	Partially desulfonated sodium lingosulfonate	Separation
Maprofix NEU	Technical sodium lauryl sulfate	Satisfactory
Maprofix	Triethanolamine lauryl sulfate	Satisfactory
Maypon 4-C	A protein condensation with lauroyl-chloride	Satisfactory
Maypon K	A salt of protein condensation with oleic fatty acid	Satisfactory
Maypon Super K	A protein condensation with coconut fatty acid chloride	Satisfactory
Mapromal	Sulfonated boric ester of oleyl alcohol	Satisfactory
Nacconol FSNO	Alkyl aryl sulfonate	Grainy
Nacconol NR	Alkyl aryl sulfonate	Separation
Nopco 1067	Alkyl naphthalene sulfonate	Separation
Nopco 2031	Sulfated hydroxy stearic acid	Satisfactory
Orvus WA Flakes	Sodium alkyl sulfate	Satisfactory
R.W.A. 300	Monobutyl biphenyl sodium monosulfonate	Grainy
Santomerse No. 1	Alkyl aryl sodium sulfonate	Grainy
Santomerse D	Decylbenzene sodium sulfonate	Unsatisfactory
Sarkosy NL-100	Sodium lauroyl sarcosinate	Satisfactory
Sequestrene AA	Ethylene diamine tetraacetic acid	Separation
Sequestrene NA-3	Disodium ethylene diamine tetraacetate dihydrate	Separation
Sipon ES	Sodium salt of lauryl ether sulfate	Satisfactory
Stepanol ME dry	Sodium lauryl sulfate	Satisfactory
Stepanol WA	Sodium lauryl sulfate	Satisfactory
Sulfatate BL	Alkyl aryl sodium sulfonate	Separation
Tergitol 08	Sodium sulfate derivative of 2-ethyl 1-hexanol	Grainy
Tergitol 4	Sodium sulfate derivative of 7-ethyl-2-methyl 4-undecanol	Unsatisfactory
Tergitol 7	Sodium sulfate derivative of 3,9-diethyltridecanol	Grainy
Tergitol EH	Sodium 2-ethylhexene sulfonate	Grainy
Trepanol T-100-T	Salts of tridecanol polyglycol ether sulfate	Grainy
Trepanol WAT	Triethanolamine salt of lauryl sulfate	Satisfactory
Triton X-200	Sodium salt of alkyl aryl polyether sulfonate	Grainy
Triton X-301	Sodium salt of alkyl aryl polyether sulfate	Separation
Triton 770 Conc.	Sodium salt of alkyl aryl polyether sulfate	Grainy
Ultrawet 30-DS	Alkyl benzene sodium sulfonate	Separation
Ultrawet 60-L	Alkyl benzene sulfonate of an organic salt	Satisfactory
Xynomine	Sulfonated fatty acid condensate	Satisfactory
	Cationic Surfactants	
Acidol 25 A	Salt of fatty acid tertiary amine	Separation
Aerosol C-61	An ethanolated alkyl guanidine amine complex	Satisfactory
Alkaterge A	Substituted oxazolines	Separation
Ammonyx CO	Alkyl dimethyl amine oxide	Separation

TABLE I. (*Continued*)

Tradenames[a]	Chemical Designation	First Screening Evaluation
Armeen O	Primary amine	Separation
Armeen 2-HT	Di-n-alkyl amine	Grainy
Armac 8-D	Acetic acid salt of n-alkyl amines	Separation
B.T.C. 100%	Alkyl dimethylbenzene ammonium chloride	Satisfactory
Catanac SN	Stearamidopropyl dimethyl β-hydroxyethylammonium nitrate	Satisfactory
Catiosan T	Mixture of complex amide condensation product and a quaternary ammonium compound	Satisfactory
Cationic SP	Stearamido isopropyl dimethyl β-hydroxy ethyl ammonium chloride	Satisfactory
Ethomeen C/25	Ethylene oxide condensation product of the primary fatty amines	Satisfactory
Ethomeen S/15	Ethylene oxide condensation product of the primary fatty amines	Satisfactory
Ethomeen T/25	Ethylene oxide condensation product of the primary fatty amines	Satisfactory
Intracol OA	Long chain fatty acid amide containing multiple amine group	Satisfactory
Isothan DL-1	Lauryl isoquinolinium bromide	Satisfactory
Katapol PN-430	Alkyl polyoxyethylene glycol amine	Separation
Lupomine	Alkylolamide	Separation
Negamine 142A	Amine ester of a long chain fatty acid	Separation
Nopco 2125	Polyoxyethylene amide condensation	Unsatisfactory
Nopcogen 11-L	Lauric alkylolamine	Separation
Nopcogen 14-L	Lauric alkylolamine condensate	Satisfactory
Nopcogen 16-L	Lauric polyamine condensation	Separation
Nopalcol BL-18	Polyoxyethylene lauric amido amine	Satisfactory
Onamine RO	A high molecular weight tertiary amine	Grainy
Quatronyx	Alkyl dimethyl carboxy methyl ammonium chloride	Satisfactory
Sapamine MS Conc.	Quaternary ammonium composition	Grainy
Triton X-400	Stearyl dimethyl benzyl ammonium chloride	Separation

[a] J. W. McCutcheon's "Surfactants Listed," Fourth Revision of Synthetic Detergents and Emulsifiers may be consulted for suppliers of the surface-active agents employed in this study.
[b] Grainy, grainy particles were observed when the ointment base was spread on the back of the hand or was rubbed between the thumb and the first finger.
[c] Separation, partial separation of either oil or water was observed.
[d] Satisfactory, a physically stable, white, smooth emulsion product was obtained.
[e] Unsatisfactory, any one or more of the following observations were made: (*a*) the ointment base produced had too thin a consistency; (*b*) a colored or off-white product was obtained; (*c*) separation of the oil and water phase occurred within two weeks of manufacture.

bility, general use, low toxicity, and availability. The anionic and cationic surfactants selected for this study are listed alphabetically with their tradenames and chemical designation in Table I. These surfactants were subjected to a preliminary screening to determine which surfactants warranted a complete evaluation in the hydrophilic ointment base.

First Screening Evaluation

The purpose of this procedure was to determine whether an ointment could be prepared using the surfactants in Table I in place of sodium lauryl sulfate and in the same concentration (1%) in the formula for hydrophilic ointment U. S. P. XVI. A Lightnin model V-7 portable electric stirrer, connected to a variac set at 90 v., was used to stir the ointment samples which were prepared according to official directions. The ointments were placed in 4-ounce green-glass ointment jars which were filled completely and were stored at 25 ± 3° for two weeks. The ointments were then visually evaluated according to the results of Table I. Twenty-nine anionic and 13 cationic surfactants produced satisfactory ointment base emulsion products based on the first screening (see Table I).

Second Screening Evaluation

The twenty-nine anionic and 13 cationic surfactants which passed the first screening test were used in a 1% concentration to prepare new hydrophilic ointment samples. The pH of the aqueous phase of the ointment base was determined using a Beckman model N pH meter. One-ounce green-glass ointment jars were completely filled and were stored in an oven at 39 ± 1°, in a refrigerator at 5 ± 2°, and at room temperature at 25 ± 3° for forty days (10). A compatibility study was simultaneously carried out by adding 3 ml. of 10% hydrochloric acid, 3 ml. of 10% sodium hydroxide solution, and 3 ml. of distilled water to separate 30-Gm. portions of each ointment base. Ointment samples containing Whitfield's combination and 10% aluminum chloride solution (25%) were also prepared. These ointment samples were stored at 25 ± 3° for forty days at which time they were visually evaluated for spreadability and washability. Spreadability was studied by spreading each ointment on the back of the hand and on a glass slab. In testing washability, the back of the hand containing a film of the ointment was placed under a faucet and water was allowed to run over it. At the same time the

TABLE II.—EFFECT OF THE SURFACTANT AND ITS CONCENTRATIONS ON THE RATE OF WATER LOSS FROM AN OINTMENT BASE

Surfactant Used in the Ointment	Per Cent Concn. of Surfactant in Ointment	Per Cent Water Loss[a] from Ointments at Intervals of Three Days						
		3	6	9	12	15	18	21
Anionic Surfactants								
Aerosol 22	1	16.35	21.09	23.77	26.23	28.99	32.13	34.59
	3	11.19	15.41	18.38	20.67	23.35	26.46	28.70
	5	7.48	10.86	13.49	15.60	17.83	20.13	21.88
Duponol LS	1	21.36	32.24	38.56	44.83	48.83	52.23	55.02
	3	12.38	17.35	21.20	26.93	31.82	36.40	40.38
	5	13.65	18.38	22.42	28.13	33.73	37.57	42.17
Igepon AP-78	1	20.55	30.12	35.64	40.78	44.04	45.86	47.90
	3	4.98	6.99	8.62	10.32	11.77	13.06	14.23
	5	6.53	9.72	11.82	13.95	15.35	16.51	17.62
Igepon T-73	1	20.33	29.69	35.25	41.11	45.53	49.59	53.30
	3	11.55	15.99	19.24	23.04	25.76	28.12	30.07
	5	10.59	14.15	17.24	21.60	25.94	29.55	32.68
Lathanol LAL	1	18.22	26.97	33.07	36.78	39.75	42.16	44.11
	3	10.22	15.45	19.94	23.36	26.28	29.41	31.89
	5	9.89	14.31	18.35	21.84	24.68	27.51	30.35
Maprofix TLS	1	12.07	16.90	20.66	23.63	26.42	29.00	31.70
	3	9.74	13.94	17.70	21.12	24.18	26.88	29.30
	5	8.30	13.23	17.65	21.45	24.86	28.03	30.89
Mapromal	1	12.59	18.17	23.01	26.71	29.92	32.79	35.29
	3	8.20	12.14	15.82	18.72	20.91	23.34	25.37
	5	8.21	12.26	16.10	19.28	22.01	24.56	27.03
Nopco 2031	1	19.26	25.80	30.11	32.57	34.71	36.62	38.19
	3	10.87	14.68	18.06	20.80	23.22	25.31	27.00
	5	11.76	15.64	19.12	22.36	25.23	27.72	29.54
Sipon ES	1	17.83	26.86	32.49	37.57	41.43	44.58	47.23
	3	14.34	20.51	24.51	28.10	31.19	34.09	36.81
	5	14.09	20.26	24.12	27.68	30.56	33.20	35.66
Stepanol ME	1	8.94	13.45	16.12	19.89	22.43	25.04	27.49
	3	9.84	18.21	23.52	27.97	31.25	34.06	36.77
	5	9.85	18.30	24.66	30.27	33.77	36.97	40.07
Cationic Surfactants								
B.T.C. 100%	1	17.49	25.33	30.39	35.38	39.21	42.85	45.86
	3	11.04	16.22	20.48	24.61	27.67	30.21	32.32
	5	9.27	12.74	16.42	19.96	22.75	25.75	27.81
Catanac SN	1	11.07	16.43	19.48	22.10	24.30	25.99	27.57
	3	4.10	6.01	6.85	7.69	8.44	9.10	9.79
	5	2.31	3.64	4.54	5.43	6.24	6.95	7.68
Ethomeen C/25	1	15.08	19.56	23.06	26.27	29.16	31.10	33.22
	3	10.33	13.43	15.93	18.04	20.12	21.56	23.27
	5	10.18	13.65	16.27	18.38	20.57	22.21	24.11
Ethomeen S/15	1	6.37	9.49	11.75	13.75	15.84	17.14	18.33
	3	3.11	5.21	6.77	7.69	8.62	9.28	10.03
	5	1.30	2.89	4.41	5.43	6.36	7.19	8.13
Ethomeen T/25	1	12.19	17.14	21.03	24.75	28.16	30.56	33.13
	3	5.91	9.54	12.87	15.88	18.34	20.44	22.63
	5	2.86	5.81	8.50	10.94	13.14	15.25	17.28

[a] Each reading presented in this table is the average of two readings.

hand was gently rubbed with the first finger. The results of spreadability and washability were reported simply as "Positive"[1] and "Negative"[2] A relative method for the evaluation was made by adding the total number of positive results obtained for the ointments containing each surfactant. The surfactants which had a total of 18 positive results were subjected to a further complete evaluation. Ten anionic and five cationic surfactants were selected for the third evaluation (see Table II).

[1] Positive. Spreadability, the ointment spreads easily forming an even layer on the skin; washability, the ointment film was readily washed from the skin with water within thirty seconds.

[2] Negative. Spreadability, the ointment resists even spreading or rolls up and does not form an even layer; washability, the ointment film is not washed away within thirty seconds.

Third Screening Evaluation

The purpose of this procedure was to evaluate the 15 remaining surfactants thoroughly for their ability to produce a satisfactory hydrophilic ointment base. Concentrations of the surfactants, 1, 3, and 5%, were used in the preparation of the ointments. In order to use more than 1% of the surfactant and to maintain the total weight of the formula, the quantity of one or more of the ingredients in the formula was reduced. This reduction was distributed equally between the stearyl alcohol and white petrolatum in the same manner as Lesshafft and DeKay (10). The amount of water in the formula was not changed since the effect of surfactant concentration on water loss from a hydrophilic ointment base was

to be studied. One-ounce tightly closed ointment samples were stored in an oven at 39 ± 1°, in a refrigerator at 5 ± 2°, and at room temperature at 25 ± 3° for forty days.

Consistency Study.—At the end of the storage period, the consistency of the ointment samples which were stored in the oven and in the refrigerator was compared to the respective samples stored at room temperature. A Schaar-Labline penetrometer[4] was used to measure the consistency of the ointments (10, 11). The method of determining the consistency was the same as the U. S. P. method for determining the consistency of petrolatum (14). The penetration measurements were made in the same 1-ounce ointment jars in which the ointments were stored. The jars were kept at room temperature at 25 ± 3° for twenty-four hours before the test was carried out.

Most of the refrigerator samples had a smaller variation (1 to 38 units[5]) in consistency than the oven samples when compared to those of room temperature. The refrigerator samples showed an increase in consistency except in the case of ointments prepared with Duponol LS, Igepon T-73, 3 and 5% concentrations of B.T.C., and 1 and 5% concentrations of Ethomeens, which showed a decrease (2 to 13 units) in consistency.

The consistency of the oven samples showed an increase of 1 to 121 units in most of the cases as compared to samples stored at room temperature. However, ointments containing Stepanol ME and 5% Aerosol 22 showed a reduction of 9 to 29 units. A slight separation was observed in the oven samples containing 3 and 5% Ethomeens. The results of this study showed no apparent relationship between the concentration of the surfactant used in ointments and the variation in consistency. This evaluation indicates the relative physical stability of ointment samples at high and low temperatures.

The oven and refrigerator ointment samples prepared using 1, 3, and 5% concentrations of Maprofix TLS, Lathonol LAL, Sipon ES, Stepanol ME, and Catanac SN, varied only slightly in consistency (less than 30 units) from the samples stored at room temperature.

Spreadability, Washability, and pH.—At the end of the storage period all of the ointment samples had positive spreadability on a glass slab, on the back of the hand, and had positive washability except the samples prepared with 3 and 5% concentrations of Nopco 2031 and B.T.C. The oven samples prepared with 3 and 5% concentrations of Ethomeens separated slightly in the bottom of their containers.

The pH of the aqueous phase of the ointments containing anionic surfactants was 6.1 to 8.8 with the exception of agent B.P.E. and Nopco 2031, which had a pH of 5.20 and 5.50, respectively. In the case of the cationic surfactants, the pH of the aqueous phase containing B.T.C., Catiosan T, Catanac SP, and Quatronyx was 3.6 to 5.2, while the pH range of the rest of the solutions were 6.1 to 9.4.

Water Retention.—The method used for the determination of water loss was similar to that used by Henny, *et al.* (13), with some modifications in temperature and relative humidity. A saturated solution of magnesium chloride ($MgCl_2 \cdot 6H_2O$) produces a relative humidity of about 32% in a closed container at temperatures from 30 to 40° (15). This solution was placed in a 1-L. evaporating dish at 39 ± 1°, which maintained the relative humidity of 32 ± 1% throughout the experiment, as indicated by a model 201 hygrometer.[6] The 1-ounce ointment jars containing ointment samples were weighed on a chainomatic analytical balance at intervals of seventy-two hours during a period of twenty-one days. The per cent water loss from the ointment bases during this period is reported in Table II.

The ointments containing 1, 3, and 5% concentrations of Stepanol ME (sodium lauryl sulfate) were used as control ointment samples for the ointments containing corresponding concentrations of a surfactant. The amount of water loss increased with an increase in concentration of surfactant in the three control samples. All of the ointment samples containing a 1% concentration of each anionic surfactant (except a control sample of 1% Stepanol ME) showed a higher water loss than the corresponding samples containing 3 and 5% concentrations. The water loss from ointments containing 3 and 5% surfactant concentrations varied according to the type of the surfactant used. The ointments containing Aerosol 22, Lathanol LAL, and Sipon ES showed a decrease in water loss with an increase in surfactant concentration, while the control ointments prepared with stepanol ME showed the opposite effect. The ointments containing Duponol LS showed the highest loss of water among all of the ointment bases.

In the case of the ointments containing cationic surfactants, the amount of water loss decreased with an increase in concentration of surfactant except for ointments containing Ethomeen C/25. The ointment prepared with a 3% concentration of Ethomeen C/25 showed a slight decrease in water loss compared to the ointment containing 5% of the surfactant. Ointments containing Catanac SN and Ethomeen S/15 showed the least amount of water loss among all of the ointment bases studied. Ointments prepared with cationic surfactants (except B.T.C.) showed less water loss than the ointments prepared with anionic surfactants. The results of this study indicate that the ionic type of surfactant does affect the water loss from an ointment base and that the concentration and surfactant composition within each ionic clasification are also factors in the water loss phenomena.

An additional ointment sample containing 2% Sipon ES was prepared which was found to be better in spreadability, washability, and consistency than the corresponding samples containing 1, 3, and 5% concentrations. Based on the screening procedures to this point the following five surfactants in the concentrations shown below were selected as warranting further study: Lathanol LAL, 3.0%, anionic; Maprofix TLS, 3.0%, anionic; Sipon ES, 2.0%, anionic; Stepanol ME, 1.0%, anionic; Catanac SN, 3.0%, cationic.

Compatibility Evaluation of Ointment Bases.—The five ointment bases which were considered the

[4] Labline Inc., Chicago, Ill.

[5] The consistency is reported as the depth, in tenths of a millimeter, of the standard cone during a period of five seconds.

[6] Serdex Inc., 91 Cambridge St., Boston 14, Mass.

TABLE III.—EVALUATION OF OINTMENT BASES FOR THEIR COMPATIBILITY WITH VARIOUS INGREDIENTS

Name of Surfactant in Ointment	Test[a] Observed on Ointments	Name of the Ingredients[b] Mixed with Ointment Bases												Total Numbers
		1	2	3	4	5	6	7	8	9	10	11	12	
Maprofix TLS	S.O.S.	1	1	2	1	1	1	1	3	1	1	3	1	
3.0%	S.O.H.	1	1	1	1	1	1	1	2	1	1	3	1	63
	Wash	1	1	1	1	1	3	1	1	1	1	3	1	
	Color	1	2	1	1	1	1	1	1	2	2	1	1	
Lanthanol LAL	S.O.S.	3	1	1	1	1	1	1	1	3	1	2	1	
3.0%	S.O.H.	3	1	2	1	1	1	1	1	2	1	2	1	63
	Wash	3	1	2	1	1	1	1	1	1	1	2	1	
	Color	1	2	1	1	1	1	1	1	1	2	1	1	
Sipon ES	S.O.S.	1	1	1	1	1	1	1	1	1	1	1	1	
2.0%	S.O.H.	1	1	1	1	1	1	1	1	1	1	1	1	55
	Wash	2	1	2	2	1	2	2	1	1	1	1	1	
	Color	1	2	1	1	1	1	1	1	1	2	1	1	
Stepanol ME	S.O.S.	1	1	2	1	1	1	2	1	1	1	2	1	
1.0%	S.O.H.	1	1	2	1	1	1	1	1	1	1	1	1	58
	Wash	1	1	1	1	1	2	1	1	1	1	3	1	
	Color	1	2	1	1	2	1	1	1	1	2	1	1	
Catanac SN	S.O.S.	1	1	2	1	1	1	1	1	3	3	1	1	
3.0%	S.O.H.	1	1	1	1	1	1	1	1	3	1	1	1	61
	Wash	1	1	1	1	1	1	1	1	3	2	1	1	
	Color	1	2	1	1	1	1	1	1	3	1	1	1	

[a] Test observed on ointments—S.O.S., spreadability on a slab; S.O.H., spreadability on the back of the hand; Wash washability; Color, change in color.

Spreadability and Washability—Group 1, all of the ointment samples stored at room temperature, in an oven and in a refrigerator had positive spreadability and washability. Group 2, one of the three samples stored at three different temperatures had negative spreadability and washability. Group 3, either two or all three of the samples stored at three different temperatures had negative spreadability and washability. Change of Color in the Ointment Bases—Group 1, No change in color was noticed in the ointment samples stored at room temperature and in an oven, as compared to the refrigerator sample. Group 2, One of the two samples stored at room temperature and in an oven showed a color change. Group 3, both the sample stored at room temperature and in an oven showed a color change as compared to the refrigerator samples.

[b] Name of the Ingredients—

	Name	Per Cent Used
1.	Ammoniated mercury	5.0
2.	Coal tar	5.0
3.	Benzoic acid	6.0
	Salicylic acid	3.0
4.	Phenol	2.0
	Glycerin	2.0
5.	Sulfur	10.0
6.	Zinc oxide	20.0
7.	Zinc undecylate	20.0
	Undecylic acid	5.0
8.	Sulfathiazole	5.0
9.	Iodine	4.0
	Potassium iodide	4.0
	Glycerin	12.0
10.	Ichthammol	10.0
11.	Aluminum chloride solution 25%	10.0
12.	Control ointment without any ingredient	

most satisfactory, were further evaluated for their compatibility with 11 selected drugs. The ointment bases were mixed with each of the drugs in the concentrations mentioned in Table III. A sample of each ointment was stored for forty days in an oven at 39 ± 1°, in a refrigerator at 5 ± 2°, and at room temperature at 25 ± 3°. At the end of the storage period, the ointment samples were examined for spreadability on a glass slab and on the back of the hand, and for washability, as described in the second screening procedure. The effect of storage at different temperatures on the color and above mentioned physical properties is shown in Table III.

As can be seen from Table III, the ointment samples prepared with Sipon ES produced the lowest total number of points and was considered to be the best among the five ointment bases. The ointment samples prepared wiith Stepanol ME totaled 59 points and was second in the group. The ammoniated mercury, Whitfield's combination, phenol, zinc oxide, and zinc undecylate ointment samples of Sipon ES stored in an oven, left a slight greasy layer on the back of the hand during the washability test, but the corresponding samples stored at room temperature and in a refrigerator showed a positive spreadability and washability. The ointment containing the cationic surfactant, Catanac SN, exhibited positive spreadability and washability in all of the samples except those containing iodine and ichthammol. The iodine ointment of Catanac SN separated in a mortar during the mixing operation, probably due to the interaction of iodine with the strong cationic portion of the dissociated cationic surfactant. The aluminum chloride ointments of Sipon ES and Catanac SN showed excellent spreadability and washability, while the other three ointment bases produced poor aluminum chloride ointments. No change in color resulted between ointment samples stored at room temperature or in the refrigerator, but discoloration was noted with a few of the oven samples.

At the end of this study it was concluded that the ointment base prepared with a 2% concentration of the anionic surfactant, Sipon ES (Sodium lauryl ether sulfate), produced the most satisfactory hydrophilic ointment based on compatibility with representative topical drugs and pharmaceutical elegance. The formula of this ointment base is given below:

Sipon ES	2.00 Gm.
Stearyl alcohol	24.50 Gm.
White petrolatum	24.50 Gm.
Distilled water	37.00 Gm.
Propylene glycol	12.00 Gm
Methylparaben	0.025 Gm.
Propylparaben	0.015 Gm.

SUMMARY AND CONCLUSIONS

1. Sixty-two anionic and twenty-eight cationic surfactants were subjected to two screening procedures to determine which surfactants warranted a complete evaluation in the hydrophilic ointment formula. At the end of the second screening procedure, ten anionic and five cationic surfactants were retained for further complete evaluation.

2. The hydrophilic ointments prepared with one, three, and five per cent concentrations of each of 15 screened and selected surfactants were evaluated for stability, spreadability, washability, consistency, and water loss. It was found that the ionic type, chemical type, and the concentration of the surfactant were factors in the water retention property of the ointment base.

3. Ointments prepared with four anionic surfactant (Lathanol LAL 3.0%, Maprofix TLS 3.0%, Sipon ES 2.0%, and Stepanol ME 1.0%) and one cationic surfactant (Catanac SN 3.0%), used in the concentration mentioned in parenthesis were found to be superior among the 15 ointment bases studied.

4. The above five ointment bases were examined for their compatibility with various drugs used in dermatological practice. The ointment base prepared with a two per cent anionic surfactant, Sipon ES, produced the most satisfactory hydrophilic ointment based on compatibility study and pharmaceutical elegance.

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Study of Anionic and Cationic Surfactants in a Hydrophilic Ointment Base II

The Effect of the Surfactant and Its Concentration on Medicament Release

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Five hydrophilic ointment bases prepared with one cationic and four anionic surfactants were studied for their medicament release characteristics using three *in vitro* methods: (*a*) a radioactive isotope, (*b*) a bacteriological, and (*c*) a physicochemical method. A modified physicochemical method has been developed to increase the sensitivity and the speed of the color zones produced per unit time. A comparative discussion of the three *in vitro* methods has been presented. The ointment sample prepared with two per cent of the anionic surfactant, sodium lauryl ether sulfate, produced the most satisfactory hydrophilic ointment, based on both physical properties and medicament release. Increasing the concentration of surfactant retarded the release of medicaments in all of the ointment bases studied with all three *in vitro* methods. Excellent correlation of results between the three *in vitro* methods was obtained.

THE RELEASE of medication from an ointment base is an important criterion in determining the therapeutic efficacy of that base. Several *in vitro* methods have been devised to estimate the rate of release of medicament from ointment bases (1–7). *In vitro* methods are of limited value in such studies, but they are a means of assessing the rate and extent of medicament release from an ointment base under the condition of the test. The *in vitro* methods which have been reported are of a comparative nature, and most are

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empirical which prevents results from being compared with those from other techniques. *In vitro* methods have been devised to study the diffusion of material through a membrane or through an agar gel using chemical, bacteriological, or radioactive tracer methods (8). The radioactive tracer techniques which have been introduced to evaluate drug release from topical preparations probably offer the most accurate and sensitive means of *in vitro* evaluation currently available.

Barker, *et al.* (3), modified the agar cup-plate method to determine the release of I^{131} incorporated in hydrophilic ointment bases. They showed that the hydrophilic ointment U. S. P. XIV in which sodium lauryl sulfate was used, had a greater release of medication than the other ointment bases prepared from the nonionic surfactants studied. Stark, *et al.* (2), designed a new apparatus for an *in vitro* method employing radioactive isotopes to study the effect of some anionic, cationic, and nonionic surfactants on the release of medicament from hydrophilic ointment bases.

The primary purpose of this study was to compare the release of medicament from five selected hydrophilic ointment bases suggested in the previous study (9) and to select the most satisfactory ointment base. A comparative study of three *in vitro* methods, (*a*) a radioactive isotope technique, (*b*) a bacteriological procedure, and (*c*) a physicochemical method, for the release of medication from ointment bases was carried out to determine the relative superiority of the emulsified bases. The results obtained by these methods were correlated to determine the relative efficiency of each method.

EXPERIMENTAL

Five hydrophilic ointment samples prepared with one cationic and four anionic surfactants were prepared according to the procedure outlined in the previous study (9). The surfactants and concentrations used were as follows: Lathanol LAL, 3%, anionic; Maprofix TLS, 3%, anionic; Sipon ES, 2%, anionic; Stepanol ME, 1%, anionic; Catanac SN, 3%, cationic.

Radioactive Isotope Method.—The *in vitro* dialysis method developed by Stark, *et al.*, was used in conjunction with a radioactive isotope (2). The general procedure and the apparatus used for this study were the same as that used by Stark, *et al.*, but significant changes were made in the temperature control device and in the counting instrumentation to provide a continuous record of the rate of release of the isotopically labeled compound from ointment bases.

In order to conduct all experiments at a uniform temperature, the apparatus was placed in a thermostatically controlled constant temperature bath. The bath, a copper tank of 2-L. capacity, was heated by a 125 W knife type immersion heater.[1] The temperature was controlled by a model 4-235 bimetallic thermostat,[2] which maintained the temperature of the bath at 37 ± 1°. The water in the bath was circulated by an electric stirrer connected to a variac set at 52 volts.

Visking C-125 seamless regenerated cellulose tubing[3] was selected as the artificial membrane (10). Sodium radioiodide (I^{131}) was chosen for use in this investigation. This isotope has a convenient half-life of eight days (11) and a suitable energy spectrum (12) to penetrate the glass wall of the apparatus housing the bismuth cathode counter.

A Model 1620 analytical count rate meter[4] was used to convert the random occurring pulses originating in a bismuth cathode side-window counting tube[5] into average counts per minute. This type of device is more convenient for the continuous observation of a dialyzing compound as the change in count rate is more quickly indicated than with a regular scaler. A second advantage of this instrument is the ease of adaptation of the rate meter to recording instruments, which provides a permanent record of the changes in activity. An Esterline-Angus model AW milliammeter graphic instrument[5] was used in conjunction with the counting rate meter to provide a permanent record of the changes in activity. The record chart[5] number 4309–X was used to record a continuous count rate *versus* time. The recording circuit is accurate within 1.5% of full-scale reading.

The membrane was attached to the dialysis apparatus as described by Stark, *et al.*, and the magnetic stirring bar was placed in the stirring compartment. The magnetic stirrer was connected to a variac set at 90 v., to maintain a constant revolving speed of the bar in each determination. Physiological salt solution (13) was added to a predetermined level on the counter-housing equivalent to a height of 105 ml.[6] The apparatus was placed in the constant temperature bath and the rate meter, set at 0–300 scale, was started to record the background. Thirty grams of labeled ointment base was prepared by mixing 0.3 ml. of I^{131} solution corresponding to approximately 30,000 counts per minute per 100 λ, with 29.7 Gm. of ointment base. The ointment base was standardized as reported by Stark. A 50 × 65 mm. Lucite sample holder containing a known amount of labeled ointment base was inverted on the membrane so that the ointment base contacted the membrane. A 50-Gm. weight was placed on the aluminum foil backing of the holder to produce a continuously uniform contact of the ointment and membrane throughout the test. The rate meter was immediately switched to 0–3,000 scale and the Esterline-Angus recorder was placed in motion. The speed of the chart on the recorder was kept at 12 inches per hour throughout all of the determinations.

Four individual release studies were made from each ointment sample to measure the variation be-

[1] Central Scientific Co., Chicago, Ill.
[2] American Instrument Co., Silver Spring, Md.
[3] The Visking Corp., Chicago 38, Ill.
[4] Nuclear Chicago Corp., Chicago, Ill.
[5] Esterline-Angus Co., Indianapolis, Ind.
[6] This volume was found suitable to produce a slight positive pressure on the membrane.

tween the four observations, and an average per cent release was calculated. The average activity of the ointment sample at each time interval was determined from the Esterline-Angus chart, which was corrected for background and then for decay correction factors over the four-hour counting period. A graph showing the per cent release of radioiodide ion from the five ointment bases *versus* time is shown in Fig. 1.

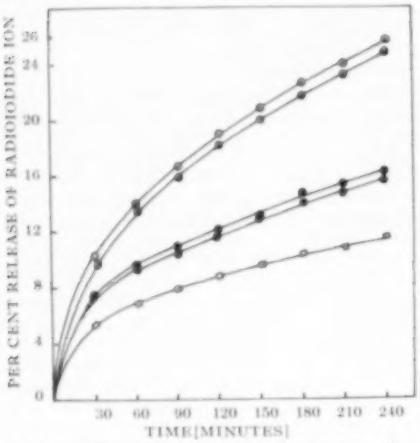

Fig. 1.—The effect of ionic surfactants on the release of radioiodide ion from a hydrophilic ointment base formula. ⊙ Sipon ES, 2.0%; ◒ Lathanol LAL, 3.0%; ● Maprofix TLS, 3.0%; ⊕ Stepanol ME, 1.0%; □ Catanac SN, 3.0%.

The results obtained at the end of this study showed that the ointments containing anionic surfactants showed a higher per cent release of radioiodide ion than the ointment containing the cationic surfactant (Catanac SN). The possible explanation of these findings is that the strongly charged anionic portion of the dissociated anionic surfactant may produce a repulsive force with similarly charged anionic iodide ion present in the ointment and thus produce a greater release of iodide ion through the membrane. Ointments containing 3% Maprofix TLS and 1% Stepanol ME produced almost identical release at the end of four hours. However, these ointments produced a lower per cent release than the ointments containing 3% Lathanol LAL and 2% Sipon ES. Based on the fact that the ointment containing 2% Sipon ES exhibited the maximum per cent release of iodide ion among the five ointment bases of the series, the effect of the release of radioiodide ion from ointment bases containing various concentrations of Sipon ES was studied (see Fig. 2).

The results of Fig. 2 suggest that the surfactant concentration significantly affects the per cent of drug release, and that for maximum release of anion from this anionic surfactant produced ointment vehicle the minimum concentration of surfactant should be used.

Bacteriological Method.—The five ointment bases and the four concentrations of Sipon ES were tested for the release of medicament using the official

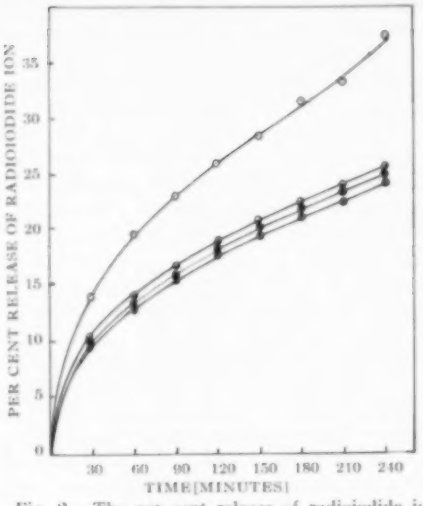

Fig. 2.—The per cent release of radioiodide ion from a hydrophilic ointment base containing various concentrations of Sipon ES. ⊙ Sipon ES, 1.0%; ◒ Sipon ES, 2.0%; ● Sipon ES, 3.0%; ⊗ Sipon, ES, 4.0%.

bacteriological method (5). In a preliminary experiment, it was observed that ointments containing 3% salicylic acid produced clear and distinct zones of inhibition. The agar plates were seeded with *Micrococcus pyogenes* var. *aureus* 209 P,[7] and four uniform agar cups were made according to the official agar-plate method. The agar cups were filled completely with the aid of a 10-ml. glass syringe. Each cup was filled with 200 ± 5 mg. of the ointment sample. Two plates, each containing four samples of each ointment base incorporated with salicylic acid, were used for the study of the release of medicament. The control plates were prepared to check the contamination or the bacterial inhibitory action of the surfactant. The plates were placed in an inverted position in an incubator at 37° for a period of twenty-four hours. At the end of the incubation period, the diameters of the zones of inhibition were measured by placing the inverted plate on a Darkfield Quebec colony counter.[8] The two perpendicular diameters for each sample of ointment base were averaged, and the mean diameter for the eight samples of each ointment base was calculated. This was converted into the area of the zone of inhibition which was corrected by substracting the area of the ointment sample.[9] The remaining value was the area of actual zone of inhibition. The results of the zones of inhibition produced by the ointments are reported in Tables I and II.

The general pattern of the release of salicylic acid from all of the ointment bases as indicated by the bacteriological method was the same as that obtained with I^{131} and the radioactive isotope method. The ointment containing 2% Sipon ES exhibited the

[7] Obtained from the Bacteriology Department of Purdue University, Lafayette, Ind.
[8] American Optical Co., Buffalo, N. Y.
[9] The area of the ointment sample, 50.28 sq. mm.

TABLE I.—ZONES OF BACTERIAL INHIBITION PRODUCED BY OINTMENT BASES CONTAINING 3% SALICYLIC ACID

Name of the Surfactant and Per Cent Concn. in Ointment	Average[a] Diameter of Zone of Inhibition, mm.	Area[b] of Zone of Inhibition, sq. mm.
Maprofix TLS 3.0%	21.28	305.54
Lathanol LAL 3.0%	23.16	371.20
Sipon ES 2.0%	23.75	392.91
Stepanol ME 1.0%	20.75	288.03
Catanac SN 3.0%	17.97	203.45

[a] Average of eight readings for each ointment base.
[b] The area of the zone of inhibition has been corrected for the area of the ointment sample, 50.28 sq. mm.

TABLE II.—ZONES OF BACTERIAL INHIBITION PRODUCED BY SALICYLIC ACID IN HYDROPHILIC OINTMENT BASES PREPARED WITH VARIOUS CONCENTRATIONS OF SIPON ES

Per Cent Concn. of Sipon ES in Ointment	Average[a] Diameter of Zone of Inhibition, mm.	Area[b] of Zone of Inhibition, sq. mm.
1	24.16	408.38
2	23.75	392.91
3	22.81	358.53
4	22.09	333.13

[a] Average of eight readings for each ointment base.
[b] The area of the zone of inhibition has been corrected for the area of the ointment sample, 50.28 sq. mm.

TABLE III.—ZONES OF COLOR PRODUCED BY DIFFUSION OF OINTMENT BASES CONTAINING SALICYLIC ACID

Name of the Surfactant and Per Cent Concn. in Ointment	Time, hr.	Average[a] Diameter of Zone of Color, mm.	Area[b] of Zone of Color, sq. mm.
Maprofix TLS 3.0%	1	13.16	85.81
	2	14.87	123.81
	3	16.47	163.32
	4	17.94	202.60
	12	28.72	597.84
Lanthanol LAL 3.0%	1	13.66	96.97
	2	14.81	122.05
	3	16.91	174.41
	4	18.62	222.12
	12	30.12	662.55
Sipon ES 2.0%	1	13.87	100.87
	2	15.75	144.62
	3	17.31	185.16
	4	18.97	232.46
	12	31.16	712.65
Stepanol ME 1.0%	1	12.91	80.69
	2	14.12	106.37
	3	15.62	141.41
	4	17.00	176.80
	12	27.31	535.76
Catanac SN 3.0%	1	12.44	71.32
	2	13.62	95.49
	3	14.66	118.59
	4	15.97	150.12
	12	24.91	437.29

[a] Average of eight readings for each ointment base.
[b] The area of the zone of color has been corrected for the area of the ointment sample, 50.28 sq. mm.

TABLE IV.—ZONES OF COLOR PRODUCED BY DIFFUSION OF SALICYLIC ACID IN HYDROPHILIC OINTMENT BASES PREPARED WITH VARIOUS CONCENTRATIONS OF SIPON ES

Per Cent Concn. of Sipon ES in Ointment Base	Time, hr.	Average[a] Diameter of Zone of Color, mm.	Area[b] of Zone of Color, sq. mm.
1	1	13.84	100.24
	2	15.75	144.62
	3	17.78	198.11
	4	19.37	244.53
	12	31.66	737.32
2	1	13.87	100.87
	2	15.75	144.62
	3	17.31	185.16
	4	18.97	232.46
	12	31.16	712.65
3	1	13.31	88.92
	2	15.12	129.34
	3	17.41	187.90
	4	18.56	220.39
	12	30.81	695.58
4	1	13.09	84.37
	2	15.09	128.65
	3	17.00	176.80
	4	18.41	216.03
	12	30.06	659.72

[a] Average of eight readings for each ointment base.
[b] The area of the zone of color has been corrected for the area of the ointment sample, 50.28 sq. mm.

maximum area of zone of inhibition of the five ointments of the surfactant series. In the case of ointments prepared with different concentrations of Sipon ES, the area of the zone of inhibition decreased with an increase in concentration of Sipon ES.

Physicochemical Method.—The physicochemical method, known as the colorimetric method, was originally developed by Waud and Ramsay (4). In preliminary experiments, some difficulties were observed in this method. It was difficult to produce a uniform contact of the ointment and the agar gel. The boundary of the color zone developed in the test was not distinct and clear which made the method less sensitive. The color zones produced after a one- and a two-hour period were not significantly different, making it difficult to record the readings every hour. These difficulties were kept in mind, and an attempt was made to improve and simplify this method, using a Petri dish in place of test tubes.

Ten ml. of a 1.5% solution of Bacto agar[10] was transferred to individual Petri dishes of 9-cm. diameters. Four penicillin assay cups, having an external diameter of 8 mm., were uniformly spaced on the solidified agar. Agar solution, 15 ml., was distributed evenly in each Petri dish. When the agar was solidified, 5 ml. of dilute solution of ferric chloride (50 parts of ferric chloride solution N. F. and 50 parts of distilled water) was spread evenly in each Petri dish. The excess solution of ferric chloride was poured off after five minutes. The penicillin cups were then removed by a gentle twisting action leaving four uniform cups in the agar medium. These cups were filled with the ointment samples as described in the bacteriological method. The plates were placed in a Thelco oven at 37° and the diameters of the zones of the

[10] Difco Laboratories, Inc., Detroit, Mich.

color produced were determined after each hour until the end of a four-hour period, and were again examined after twelve hours. The diameters were measured by the same technique as in the bacteriological method. The mean diameter of each ointment base was converted into the area of the color zone which was corrected for the area of the ointment sample. These results are presented in Tables III and IV. A comparative chart showing the release of salicylic acid from ointment bases using the two *in vitro* methods, (*a*) physicochemical and (*b*) bacteriological, is shown in Fig. 3.

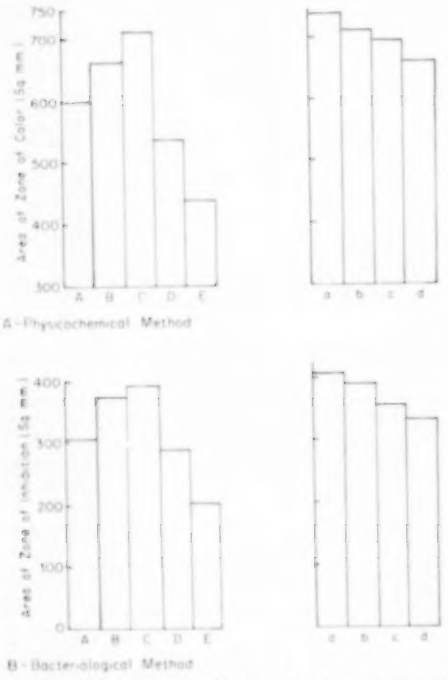

Fig. 3.—Release of salicylic acid from ointment bases by two *in vitro* methods.

A. Physicochemical method—area of zone of inhibition in sq. mm. at the end of a twelve-hour period. *A*—Maprofix TLS 3.0%, *B*—Lathanol LAL 3.0%, *C*—Sipon ES 2.0%, *D*—Stepanol ME 1.0%, *E*—Catanac SN 3.0%. *a*—Sipon ES 1.0%, *b*—Sipon ES 2.0%, *c*—Sipon ES 3.0%, *d*—Sipon ES 4.0%.

B. Bacteriological method—area of zone of inhibition in sq. mm. at the end of a twenty-four-hour period.

The surfactant or any other ingredients of the ointment did not produce any color reaction with ferric chloride solution as indicated by the control plates. The general pattern of the release of salicylic acid from all of the ointment bases was the same as that obtained by the previous two methods. Ointments prepared with anionic surfactants showed a higher release of salicylic acid than the ointment containing cationic surfactant (Catanac SN). The rate of release of salicylic acid was slowest in the case of the ointment prepared with 1% Stepanol ME among the four ointments containing anionic surfactants. The ointment prepared with 2% Sipon ES showed the highest rate of release of salicylic acid at any time during the test period. The rate of release of salicylic acid from ointment bases prepared with various concentrations of Sipon ES, decreased with an increase in surfactant concentration. Similar results were also obtained by the previous two procedures. The rate of release of salicylic acid from ointment bases prepared with 1 and 2% Sipon ES were virtually identical for the first two hours, after which the ointment sample containing 1% surfactant showed slightly greater release.

At the end of this study, it was concluded that the ointment prepared using 2% anionic surfactant, Sipon ES, was found to be the most satisfactory hydrophilic ointment base. It was compatible with a large number of common topical therapeutic ingredients, produced the most elegant pharmaceutical product as shown in the previous paper (9), and showed the maximum release of medicament as indicated by three *in vitro* methods among the five ointment bases selected.

DISCUSSION

The general pattern of the release of medicament from the five ointment bases studied remained the same in all three *in vitro* methods tested. The quantitative determination of the amount of drug released per unit of time was easily determined by the radioactive isotope method but was difficult to establish by the bacteriological and physicochemical methods. The sensitivity and accuracy of the isotope method was superior, because the release of the medicament through a membrane was recorded automatically by the counter and the recorder, while in the case of the other two methods the release of medicament was determined by the visual measurements of the zones, which undoubtedly results in some personal errors. The release of medicament during the first four-hour period was easily determined by the isotope and physicochemical methods, but was not possible for the bacteriological method because of the twenty-four-hour period of incubation.

In comparing the physicochemical and bacteriological methods, the former was simpler, less time consuming, equally accurate, less costly, and did not require aseptic technique. The bacteriological method can be used only for ointments containing antiseptic substances and the physicochemical method can be used only for ointments containing ingredients which can produce some color reaction when mixed with a suitable reagent. It is concluded that the radioactive isotope method is superior in both sensitivity and accuracy as well as in the determination of the amount of medicament which is released per unit of time. The physicochemical method was considered the second best of the three methods studied, based on its simplicity, rapidity, and fairly good accuracy.

SUMMARY AND CONCLUSIONS

1. Hydrophilic ointments prepared with one cationic (Catanac SN 3%) and four anionic (Lathanol LAL 3%, Maprofix TLS 3%, Sipon

ES 2%, and Stepanol ME 1%) surfactants used in the concentrations mentioned in parenthesis were studied for the release of medicament using three *in vitro* methods, (*a*) radioactive isotope, (*b*) bacteriological, and (*c*) physicochemical methods.

2. The general pattern of the release of medicament from all of the ointment bases remained the same when tested by these methods.

3. The ointment prepared with two per cent anionic surfactant, Sipon ES, was found to be the most satisfactory hydrophilic ointment base. The increase in concentration of Sipon ES retarded the release of medicament from ointment bases in all three *in vitro* methods.

4. A modified physicochemical method is suggested to increase the sensitivity and the speed of the color zones produced per unit of time.

5. A comparative discussion of three *in vitro* methods has been presented and it was concluded that the radioactive isotope method was superior in both sensitivity and accuracy as well as the quantitative results which can be obtained per unit of time.

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Solubilization of Anti-inflammatory Steroids by Aqueous Solutions of Triton WR-1339

By D. E. GUTTMAN†, W. E. HAMLIN, J. W. SHELL, and J. G. WAGNER

Clear solutions of anti-inflammatory steroids, which have concentrations of steroid considerably greater than the water solubilities of the steroids, are being sold commercially for ophthalmic use. The present study was undertaken to obtain quantitative data on the solubilizing power of Triton WR-1339 for three anti-inflammatory steroids. The apparent solubility of each of the steroids, prednisolone, methylprednisolone, and fluorometholone, in an aqueous solution of Triton WR-1339 was found to be linearly dependent on the per cent Triton present. Both the slopes of the linear solubility plots and the water solubilities of the steroids are, in the order, prednisolone > methylprednisolone > fluorometholone. The marked difference in the solubility of fluorometholone in aqueous solutions of Triton WR-1339 from the other two steroids having a 21-hydroxyl group indicates that an entirely different pharmaceutical problem is involved with the steroid having the type of side chain present in fluorometholone.

VARIOUS AUTHORS (1–14) have reported the use of surface-active materials, e. g., proteins, bile salts, and surfactants, to solubilize steroid hormones. For ophthalmic use it is desirable to prepare clear solutions of anti-inflammatory steroids which have concentrations of steroid considerably greater than their water solubility. The surface-active materials used in such preparations must have sufficient solubilizing power to attain the necessary steroid concentration and must be nonirritating and noninjurious to the eye. Johnson (13, 14) studied a large number of surfactants and found that both Triton WR-1339 and Tween 80 were satisfactory for preparing suitable aqueous solutions of the adrenalcortical hormones.[1]

The present study was undertaken to obtain quantitative data on the solubilizing power of Triton WR-1339 for three anti-inflammatory steroids: prednisolone, methylprednisolone, and fluorometholone.

EXPERIMENTAL

Materials.—The prednisolone was recrystallized once from alcohol-water, then dried for twelve hours

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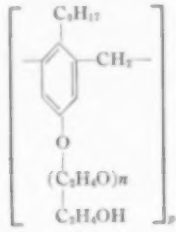
[1] Two of the commercial products are Eye Drops Optef, 0.2% and Eye Drops Neo-Deltef, 0.2%, sold by The Upjohn Co.

at 70°. The methylprednisolone and fluorometholone were micronized. The Triton WR-1339 was obtained from Winthrop-Stearns.

The structure of Triton WR-1339 according to Rohm and Haas Co. is as follows:



where $n \simeq 10$ and $p \simeq 4$. The calculated mole-equivalent weight ($n = 10$, $p = 1$) is 702.9, and the average molecular weight ($n = 10$, $p = 4$) is 2,812.

Equilibration.—*Method A.*—Excess steroid and the Triton-water solution were heated in a vial on the steam-bath for ten minutes before placing in a constant temperature bath at 25.0° where the vials were slowly rotated for two weeks. The vials were allowed to stand in the bath for one week. Some of the solubility studies with prednisolone were done this way.

Method B.—Excess steroid and the Triton-water solution were slowly rotated in 10-cc. vials in a constant temperature bath at 25° for one week. Some of the solubility studies with prednisolone were done this way.

Method C.—Excess steroid and the Triton-water solution were vigorously agitated at 37° for two days then were vigorously agitated at room temperature (approximately 25°) for two days. The samples were then allowed to stand for three days at room temperature. This method was used for the solubility studies with methylprednisolone and fluorometholone.

Assay Procedure.—*Method 1.*—All Triton-water solutions containing prednisolone were assayed for steroid by withdrawing 5 ml. of supernatant from the vial, extracting the steroid with chloroform, evaporating 2 to 10 ml. of chloroform extract to dryness, taking up the steroid residue in ethanol, and assaying the alcoholic solution by the triphenyltetrazolium color procedure (15).

Method 2.—All Triton-water solutions containing methylprednisolone or fluorometholone were assayed for steroid by determining the ultraviolet absorption spectrum of the sample solution after centrifugation using a blank prepared with the same concentration of Triton as the sample and carried through the same equilibrium procedure as the sample. The aqueous Triton-fluorometholone solutions were diluted 1:20 and the aqueous Triton-methylprednisolone solutions were diluted 1:100 with 95% alcohol. The absorption maximum was used to calculate the concentration of steroid. An absorptivity (1%, 1 cm.) of 400 was used for both methylprednisolone and fluorometholone. Calibration data indicated this absorptivity was valid since the presence of Triton at the dilutions used did not significantly change the value from that observed with 95% alcohol alone.

Method 3.—For determination of the shift in the ultraviolet absorption maximum of the steroids in aqueous-alcoholic solutions of Triton WR-1339, an aliquot of the steroid in aqueous Triton solution was diluted to 100 ml. with 95% alcohol. The ultraviolet absorption spectrum was obtained with a Cary recording spectrophotometer using a blank prepared in the same way but without steroid. Reasonably low slit widths at or near the absorption maximum with the Cary instrument indicated that distortion had not occurred despite the fact that the Triton WR-1339 also had appreciable absorption in the 230 to 250 mμ region.

RESULTS

The observed solubilities of the steroids in water and aqueous solutions of Triton WR-1339 are shown in Table I.

The apparent solubility of a steroid in an aqueous solution containing Triton WR-1339 was found to be linearly dependent on the per cent of Triton present. This relationship can be expressed by the equation

$$S = So + a\,(\text{Triton}) \qquad \text{(Eq. 1)}$$

where S = the apparent solubility of the steroid in aqueous Triton solution, So = the solubility in water, a = a constant representing the slope of the solubility curve, and (Triton) = the per cent (w/v) of Triton present.

The data of Table I are plotted in Fig. 1. The constants of the "least-squares" regression lines are shown in Table II.

Table I.—Observed Solubilities of the Steroids in Water and Aqueous Solutions of Triton WR-1339

Triton WR-1339 % w/v	Observed Solubility of Steroid, mg./ml.		
	Prednisolone	Methylprednisolone[c]	Fluorometholone[c]
0	0.223[a], 0.239[b]	0.095	0.003, 0.010
2	0.693[a]	...	...
4	1.196[a],	...	...
5	1.458[b]	...	0.065, 0.080
6	1.706[a], 1.716[b]	...	...
7	1.881[b]	...	...
8	2.219[a], 2.217[b]	...	...
9	2.481[b]	...	...
10	2.719[a], 2.719[b]	1.25	0.103, 0.090
12.5	...	1.52	0.118, 0.143
15.0	...	1.80	...

[a] Equilibration procedure was method A; assay procedure was method 1.
[b] Equilibration procedure was method B; assay procedure was method 1.
[c] Equilibration procedure was method C; assay procedure was method 2.

Table II.—Intercepts (*So*) and Slopes (*a*) of Linear Plots Shown in Fig. 1

Steroid	*So*, mg./ml.	*a*
Prednisolone	0.215	0.249
Metnylprednisolone	0.097	0.114
Fluorometholone	0.013	0.00927

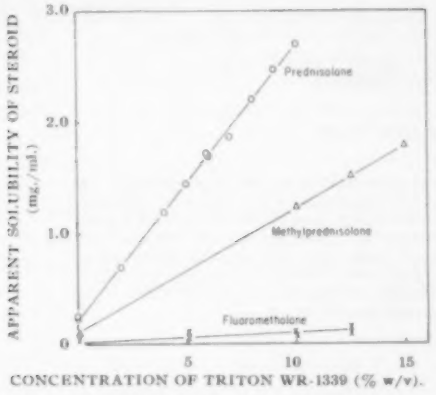


Fig. 1. The solubilities of some anti-inflammatory steroids in aqueous solutions of Triton WR-1339.

TABLE III.—MAXIMUM SOLUBILIZING POWER OF TRITON WR-1339 FOR ANTI-INFLAMMATORY STEROIDS

Steroid	Δ Steroid, mg./ml. / Δ % w/v Triton WR-1339	Moles of Steroid per Mole-equiv. of Triton WR-1339	Moles-equiv. of Triton WR-1339 per Mole of Steroid
Prednisolone	0.249	0.0486	20.6
Methylprednisolone	0.114	0.0214	46.7
Fluorometholone	0.00927	0.00173	578.0

TABLE IV.—SHIFT IN THE ULTRAVIOLET ABSORPTION MAXIMA OF THE STEROIDS IN AQUEOUS-ALCOHOLIC SOLUTIONS OF TRITON WR-1339

Triton WR-1339 % w/v	λ Max. of Fluorometholone, mμ	λ Max. of Methylprednisolone, mμ
0	243, 243.0	...
5.0	243, 243.5	...
10.0	247, 247.0	241.3
12.5	250, 251.0	243.3
15.0	254, 255.0	243.7

From the linear solubility curves we have calculated the saturation capacities of the nonionic surfactant, Triton WR-1339, for the respective steroids. These values are collected in Table III.

In aqueous-alcoholic Triton solutions the steroids investigated showed ultraviolet absorption curves that were similar to those recorded in water-alcohol. However, as the concentration of Triton increased there was a shift in the absorption maximum toward longer wavelengths. Table IV indicates these shifts in absorption maxima to longer wavelengths for fluorometholone and methylprednisolone in aqueous-alcoholic solutions of Triton.

DISCUSSION

The shifts in the absorption maxima of the steroids toward longer wavelengths as the concentration of Triton increases is similar to that reported by other authors. Similar shifts were observed in the case of solubilized polycyclic aromatic hydrocarbons (16), for various steroids solubilized in aqueous sodium lauryl sulfate (7), and for chloroxylenol solubilized in water by polyethylene glycol 1000 monoacetyl ether (17). This behavior is typical of instances where association is possible with the solvent by hydrogen bonding (3). It seems likely, therefore, that the steroids are associated with the polyoxyethylene chain of Triton WR-1339.

When the slopes, a, of the linear solubility plots of Fig. 1 and Table II are plotted against the intercepts, So, an apparent straight line is obtained. This indicates an apparent relationship between the water solubility of a steroid and its ability to be solubilized by Triton WR-1339. Although this relationship appears to be valid for the three steroids investigated, additional compounds must be studied to check the validity of the correlation for steroids as a general class. It is interesting to note that the melting points of the steroids studied fall in the order, prednisolone 232–236°, methylprednisolone 240°, and fluorometholone 292–303°. This suggests that qualitatively similar energy interchanges occur in all three equilibrium situations.

SUMMARY AND CONCLUSIONS

1. The solubilization of prednisolone, methylprednisolone, and fluorometholone by aqueous solutions of Triton WR-1339 has been studied quantitatively.

2. The apparent solubility of each steroid in an aqueous solution containing Triton WR-1339 was found to be linearly dependent on the per cent of Triton present.

3. The greater the water solubility of the steroid, the greater the solubilizing power of Triton WR-1339. Both the slopes of the linear solubility plots and the water solubilities of the steroids are in the order prednisolone > methylprednisolone > fluorometholone.

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Treatment of Experimental Streptococcal Nephritis I

A Preliminary Report

By C. K. WILLIAMSON

The role of a group A, type 12 streptococcal polypeptide in the pathogenesis of acute nephritis is confirmed. Our proposal for treatment of this disease assumed the presence of an antibody to an antigenic complex, one member of which was the nephritogenic polypeptide of the streptococcus. We postulated that specific complementary residues of the streptococcus would block the nephritogenic antibody and arrest the disease. Preliminary evidence, based on the use of either antigenic residues of the streptococcus or heated nephritogenic polypeptide, suggests that this was a valid premise.

THE ASTUTE OBSERVATION of Ophuls that particular strains of streptococci were important in producing nephritis in man, was unheeded for several decades (1). Until recently, there has been a paucity of literature bearing upon the association of type specific streptococci with nephritis. However, epidemiological evidence (2–5) and experimental evidence (6–9) reported during the last decade makes it clear that group A, type 12 streptococci are etiologically significant in glomerulonephritis. This has been further amplified by the isolation of the nephritogenic substance from type 12 streptococci by Matheson and Reed (10). It is recognized, of course, that other types of streptococci, particularly types 4 and 25, are associated with nephritis but their occurrence has been less frequent and they have been little studied.

The hypothesis that glomerulonephritis is an autoantigenic response seems untenable. The experimental approach used to confirm this hypothesis has always involved the use of a nephrogenic antibody.[1] While the methods used to produce these antikidney antibodies have varied, particularly with reference to the animal species used, the essential features of the experimental approach have been the same (11, 12). Concensus holds that the fundamental hypothesis of these studies is invalid. Reed (8) points out that their method of producing nephritis is not analogous to the mechanism of the disease in man. Vorlaender (13) concludes that, "Contrary to the theory of Cavelti, *et al.* (1945), acute glomerulonephritis is therefore not caused by an interaction of tissue autoantibodies with the own kidney, but results from the immunological interaction with foreign antigens, usually of a bacterial nature (streptococcal), rarely with nonbacterial foreign antigens (foreign proteins, serum sickness, etc.)." Finally, Ehrich, *et al.* (14), stated that although glomerulonephritis has been produced experimentally with antikidney serum, the impact of these studies on our concept of human nephritis has been negligible.

The hypothesis of Reed and Matheson, concerning the pathogenesis of glomerulonephritis, postulated that the disease was an immunological response to a new nephritogenic complex of renal protein (RP) and a nephritogenic substance (NS) elaborated by streptococci of Lancefield's group A, Griffith's type 12 (6). The experimental disease which they produced was analogous to the illness in humans in which a time lapse occurred between the streptococcal infection and the appearance of acute glomerulonephritis (6). They also showed that nephritis could be induced in rabbits either by using sterile culture filtrates of the type 12 streptococci isolated from patients ill with glomerulonephritis or by using the nephritogenic substance which these authors described as a low molecular weight polypeptide which apparently lacked antigenicity *per se*. However, it was obviously nephrotropic and modified some unknown kidney substance to produce the antigenic nephritogenic complex. Antibody produced in response to this modified renal substance provoked the acute allergic response in the host. We believed that if the antibody being synthesized could be blocked *in vivo*, this would arrest the hypersensitive reaction occurring in renal tissues.

Therefore, it was the purpose of this study to

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[1] This is frequently called a nephrotoxin but it is not actually a biological poison. The term nephrogenic antibody was suggested by Vorlaender and is used here to avoid confusion with the actual nephritogenic substance isolated from type 12 streptococcal culture filtrates by Matheson and Reed.

explore possible ways to block antibody to the so-called renal protein-nephritogenic substance (RPNS) of Reed. The experiments of Reed and Matheson emphasized that use of whole cell type 12 antigens was not a feasible desensitization approach (7). Indeed, whole cell antigens tended to aggravate the disease. Reed stated that, "There is, at present, no effective immunizing agent available against type 12 nephritogenic substance. Therefore, when cases of acute nephritis appear in a closed population group, all group A streptococcus carriers, as well as cases, should be treated at once with adequate amounts of penicillin" (8). Rammelkamp, discussing the treatment of this disease, made essentially the same recommendations (15). Unquestionably, antibiotics help to minimize the disease in patients and possibly to prevent the syndrome in contacts. The effectiveness of such an approach would be determined by how early treatment was begun since the severity of the nephritic syndrome would depend in large measure on how much NS had been elaborated by the microorganisms before they were inactivated by the treatment agent. Assuming that enough NS had been produced to indirectly provoke renal complications, then blocking Reed's RPNS antibody should modify the symptoms. Reed and Matheson's (6) proposed mechanism for the pathogenesis of acute nephritis is diagrammed below together with our own proposal for arresting this syndrome (Fig. 1).

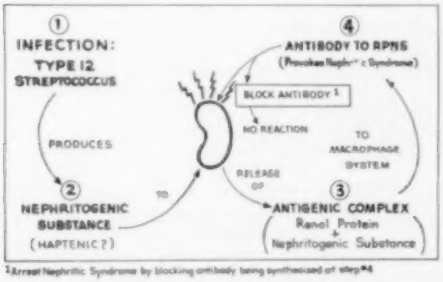

Fig. 1.—Proposed mechanism for induction of acute glomerulonephritis.

EXPERIMENTAL

Animals.—New Zealand white female rabbits, weighing four pounds, were used in all experiments. Water and a complete ration rabbit chow was provided freely. Early in these experiments a number of animals became constipated and died. The rabbits were fed fresh greens two or three times weekly to obviate this problem.

Cultures.—The type 12 streptococcus used in these experiments was very kindly provided by Dr. Roger W. Reed of McGill University. This culture was strain H8 which he isolated during the 1951 Nova Scotia epidemic (4). Organisms were maintained in the dextrose brain broth medium of Rosenow and were grown in Todd-Hewitt broth at 35° before use.

Infection.—Local infection in rabbits was induced by the subcutaneous injection of 0.2 ml. of a whole twenty-four-hour culture. Infection was terminated ten days later by three daily injections of 80,000 units of penicillin.

Filtrates.—The broth, from cultures used to infect rabbits, was clarified by centrifugation and sterilized by filtration through Seitz EK pads.

Nephritogenic Substance (NS).—This polypeptide was isolated from broth filtrates of type 12 cultures by the method of Matheson and Reed (16). The fraction precipitated by ammonium sulfate between 60 and 70% of complete saturation was used.

Peripheral Arterial Pressure.—A blood pressure apparatus, patterned after that described by Reed and Matheson (6) was used. The operator occluded blood flow at the bifurcation of the central ear artery and signaled the exact moment when blood reappeared. An assistant observed and recorded all manometric readings. Ten readings, with a maximum fluctuation of 4 mm. Hg, were taken and averaged for each blood pressure determination. Normal blood pressures for each rabbit were based on the average of readings made for five to ten consecutive days before experiments were begun.

Antigenic Residues.—The experimental biological used in these studies was a hydrolytic residue of whole streptococcal cells of the H8 strain. These "antigenic residues" were prepared according to the method of Rosenow (17). A dense suspension of the streptococci in glycerin-saturated sodium chloride solution (2:1, v/v) was diluted with saline to yield a cell concentration of approximately 10^{10} cells/ml. These diluted suspensions were autoclaved at 121° for ninety-six hours and finally adjusted to their original volume with distilled water. The suspensions, which were slightly acid (pH 5–6) prior to autoclaving and markedly acid (pH 2.5–3) after autoclaving, were adjusted to neutrality with 0.1 *N* sodium hydroxide before use. The product was then distributed in 100-ml. vaccine bottles and sterilized at 121° for fifteen minutes.

Histology.—Kidneys were placed in Baker's formal-calcium (18) to which a trace of calcium carbonate had been added. All sections were stained with hematoxylin-eosin.

Chromatography.—Analyses of the nephritogenic substance were performed to ascertain whether our isolated fraction was similar to that isolated by Matheson and Reed (16). Their procedures were therefore followed for the hydrolysis of the polypeptide and for the two dimensional paper chromatographic method.

RESULTS

Animal Studies.—Our initial experiment involved a study of six rabbits which were infected with the H8 strain. These animals developed a maximum blood pressure elevation between sixteen and twenty days later. We were reluctant to begin administration of antigenic residues (AR) until hypertension was marked and sustained. Un-

happily, we deferred treatment too long to secure unequivocal data because blood pressures of most animals began to return to normal about twenty-four hours before AR injections were begun. Yet blood pressures of control animals either began to decrease and then elevated again before finally returning to normal or else they returned to normal quite gradually. Figure 2 expresses these data in terms of the time required for blood pressures of test and control animals to return to normal. While these data were not impressive they did suggest that AR was blocking RPNS antibody. It seemed reasonable to expect that if the AR dosage and the dosage schedule were more carefully regulated, more definitive results could be obtained.

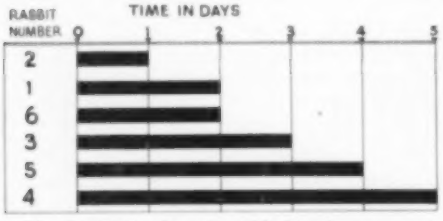

Fig. 2.—Reduction of rabbit blood pressures to normal values after treatment with antigenic residues. Rabbits No. 4 and 5 were not treated.

We elected, in our second experiment, to use the sterile broth filtrates saved from the original cultures which had been used in the previous experiment to infect the rabbits. We had also isolated a small quantity of nephritogenic substance from 1 L. of this original broth filtrate. It was enough to prepare only two rabbits but we thought it important to evoke the nephritic syndrome by the various techniques known to be effective. Rabbits No. 7 and 8 received this nephritogenic substance which represented the fraction precipitated between 60 and 70% of saturation with ammonium sulfate. Rabbits No. 9, 11, 13, 14, and 15 received injections of sterile broth filtrate from the twenty-four-hour streptococcal culture. Rabbits No. 10 and 12 died before treatment was begun. Blood pressure determinations on all of these animals were made every two or three days until the experiments had progressed beyond the seventh week. Thereafter, readings were usually taken twice weekly.

Rabbit blood pressures began to increase, in most cases, between the fifth and seventh days. Treatment with AR, which was begun in test animals on the eighth day, was repeated every forty-eight hours for the next two weeks. The precise dosage schedule is given in Figs. 3–6. The blood pressures of most test animals under treatment returned to within normal limits after forty-eight hours while the blood pressures of control animals continued to elevate and were sustained at hypertensive levels for about two months. An attempt was made to reduce the dosage of AR from 5 ml. to 2 ml. after the fifth injection, but this resulted in an elevation of blood pressure in several of the test animals. Rabbit No. 9 became difficult to control and had to be treated with additional AR. Otherwise, little difficulty was

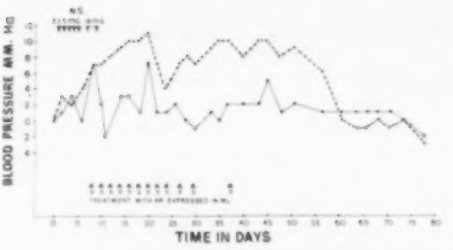

Fig. 3.—Average blood pressure readings of two rabbits receiving nephritogenic substance isolated from broth filtrates of type 12 streptococci. ——, Rabbit No. 7, treated with AR. ---, Rabbit No. 8, not treated.

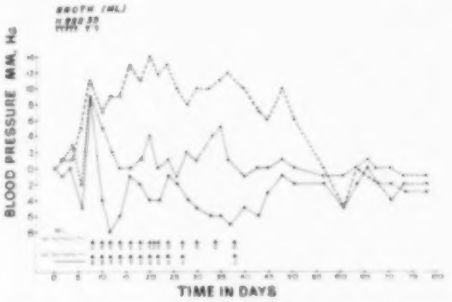

Fig. 4.—Average blood pressure readings of three rabbits receiving sterile broth filtrates of type 12 streptococci. . . ., Rabbit No. 9, treated with AR. ——, Rabbit No. 11, treated with AR ---, Rabbit No. 13, not treated.

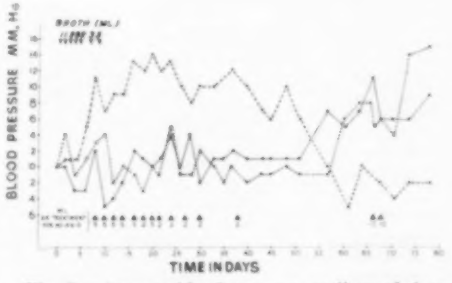

Fig. 5.—Average blood pressure readings of three rabbits receiving sterile broth filtrates of type 12 streptococci. . . ., Rabbit No. 14, treated with AR. ——, Rabbit No. 15, treated with AR ---, Rabbit No. 13, not treated.

experienced in keeping blood pressures within normal limits. However, it has been shown that after about the eighth week, blood pressures may again elevate without additional injections of NS (7). This did, in fact, occur with rabbits 14 and 15. Interestingly, neither of these animals had developed any significant hypertension until this time. Attempts to treat them with AR during this exacerbation were not successful. An attempt to explain the reason for this phenomenon will be presented

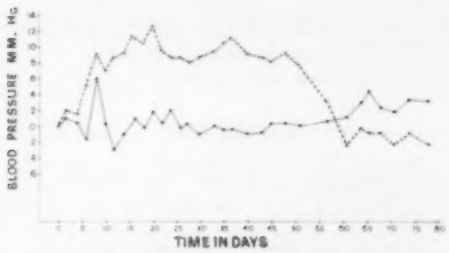

Fig. 6.—Combined blood pressure readings of all rabbits. —, Control animals, No. 8 and 13, no treatment with AR. ——, Test animals, No. 7, 9, 11, 14, and 15, treated with AR.

in the discussion. The other test animals did not become hypertensive a second time and all rabbits were sacrificed on the seventy-eighth day of the experiment.

Histology.—Histopathological evidence was equivocal. Varying degrees of pathological lesions were present in all sections and the most consistent lesion was that of tubular epithelial degeneration. Even though all animals were hypertensive at some stage in the course of these experiments it had been hoped that the results would show some differential between control and test animals. Outlined below are the various histopathological observations recorded for rabbits No. 7 through 15.[2]

No. 7.—A few glomeruli evidenced mild capsular adhesions.

No. 8.—Diffuse tubular epithelial degeneration and glomerular congestion was evidenced. Several glomeruli revealed marked capsular adhesions. Hyaline casts were present throughout the tubules. The glomeruli also evidenced coalescence of the red corpuscles.

No. 9.—Calcification quite extensive in cortex. Extensive tubular epithelial degeneration and glomerular congestion also present. Several glomeruli revealed a marked degree of cellularity.

No. 11.—Calcification in cortex also quite extensive in this section. A generalized glomerular congestion and extensive tubular epithelial degeneration was also present.

No. 13.—Diffuse glomerular congestion and tubular epithelial degeneration was present. Very few hyaline casts were observed in the tubules. Mild capsular adhesions were also evidenced in a few areas.

No. 14.—Subcapsular and cortical calcification observed, but not extensive in distribution. Diffuse tubular epithelial degeneration was present. Masses of red corpuscles appeared to have coalesced in the glomeruli. Very few hyaline casts observed in tubules.

No. 15.—Mild subcapsular and cortical calcification was present in this tissue. Diffuse and quite severe tubular epithelial degeneration and a rather mild glomerular congestion was also evidenced.

Chromatography.—The two dimensional separation of NS hydrolysates was essentially the same as that reported by Matheson and Reed (16). However, their spots 14 and 15 representing unidentified compounds could not be located.

DISCUSSION

These preliminary experiments lend confirmation to the studies of Reed and Matheson regarding the role of type 12 streptococci, and the specific nephritogenic polypeptide which they elaborate, in the pathogenesis of acute glomerulonephritis. The fact that the nephritis, produced in rabbits by sterile broth filtrates or by the nephritogenic substance isolated from broth filtrates of type 12 cultures, mimics the disease in humans following infection with these organisms is significant. Important too is the fact that NS has a molecular weight generally considered to be too low to give it antigenic status (16). However, this problem is perhaps resolved by some unknown renal substance serving as a carrier for NS. If NS complexes with renal substance it would acquire antigenic properties but it would also serve to alter the surface of the renal substance making it foreign to the host that produced it. It therefore appears unlikely that NS directly damages renal tissue but instead acts indirectly by altering a renal substance to render it antigenic to its host. Antibody produced against this complex could then evoke damage to renal tissues. Our antigenic residues apparently combine with the antibody to this complex thus blocking it from reacting with renal tissues.

The secondary elevation of blood pressures of some rabbits, alluded to earlier, has been observed by others (7). That our AR was ineffective in correcting this secondary hypertension can be attributed to use of inadequate dosages. When AR was used originally to treat the nephritic syndrome we ascribed success of this approach to a blocking action against Reed's RPNS antibody. While this achieved the desired result it served also to modify the anti-RPNS such that this antibody became antigenic (as an AR-anti-RPNS complex) in the host rabbit (19). Therefore, as treatment was continued, dosage should have been increased to make available sufficient AR to combine with anti-RPNS as well as with the antibody to the antigenic complex of AR-anti-RPNS (antiantibody). This circumstance did not become obvious until the exacerbation occurred at about the eighth week. At this time empirical treatment did not suffice to block anti-RPNS because of the excess of circulating antiantibody. Hence the dosage was inadequate to react with both antibody systems and anti-RPNS was free to react with kidney tissue.

It has not been possible as yet to determine the nature of the antigenic residues. Such hydrolytic products are obviously complex but they apparently contain components specific for the antibody to RPNS. The specific nature of the reaction occurring *in vivo* has been under investigation for several months. One observation worth citing at this time is that animals made hypertensive with nephritogenic substance precipitated at 65–70% saturation with ammonium sulfate have been successfully treated with the same fraction of NS that had been heated at 100° for one hour (20).

These findings pose multiple basic questions

[2] Grateful acknowledgment is made to Dr. Alan R. Wagner of the Warren-Teed Products Co. for these histopathological observations.

regarding, among others, the fate of NS, the chemistry of the renal substance with which it is assumed to react, the nature of the antibody produced, and the ultimate complexity of the antiantibody complexes formed. It is also hoped that these data will prompt others to speculate on the applicability of this experimental approach to other diseases regarded as infectious allergies.

SUMMARY

Experimental streptococcal nephritis has been produced in rabbits by infection with group A, type 12 streptococci, by injection of sterile culture filtrates of this streptococcus, and by use of the nephritogenic polypeptide isolated from such culture filtrates. Hydrolysates of the nephritogenic polypeptide studied chromatographically confirmed the amino acid composition of this dialyzable substance.

The presence of antibody to an antigenic complex containing nephritogenic polypeptide formed the basis for our proposal for treatment of acute glomerulonephritis. We postulated that effective blocking of such antibody would abort the nephritic syndrome. Treatment of rabbits with antigenic residues of group A, type 12 streptococci or with heated nephritogenic polypeptide lent confirmation to this premise.

Further studies are in progress.

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Sulfide Derivatives of Cysteine

By MATTHEW VERDERAME

Several new amide derivatives of L-3-carboxymethylthioalanine were prepared as possible antiviral agents by the alkylation of cysteine. None of these was found to be effective, however. Unrelated to this group, but nonetheless sulfides of cysteine, are L-3-(2-hydroxyethylthio)-alanine and L-3-benzhydrylthioalanine, which were also prepared and tested. The former showed slight activities against a poliomyelitis virus and against a *Klebsiella pneumoniae* infection in Swiss mice. The latter compound was found to be helpful in treating a trichomonas infection in hamsters.

VARIOUS SULFIDES of cysteine have been prepared in this study primarily in the hope of discovering antiviral agents. Sufficient quantities of compounds were prepared, however, to permit the carrying out of antibacterial and antimetabolite screening as well. As was reported earlier (1) in connection with this same problem, L-3-carboxymethylthioalanine showed slight activity against encephalitis-type viruses in mice. The antitubercular activity of certain ethylmercapto compounds, ethionine in particular, is, of course, well known (2). Most of the compounds prepared in this work are amide derivatives of L-3-carboxymethylthioalanine; three others do not fall into this group, but are otherwise somewhat related to ethionine.

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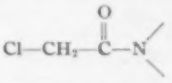
DISCUSSION

The amide derivatives of L-3-carboxymethylthioalanine were prepared by a two-step synthesis. The acylation of various amines with chloroacetyl chloride provided a batch of known intermediates, which then served as alkylating agents for the mercapto function of cysteine, according to previously employed procedures (1,3). The alkyl halides,

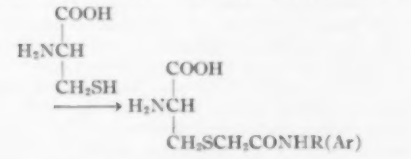
TABLE I.—MONO- AND DI-SUBSTITUTED CHLOROACETAMIDES



No.	N	Reference	Yield Reported, %	Yield Obtained, %	Modification of Known Procedure	M. P. Lit., °C.	M. P.[a] Obs., °C.
1A	Phenylamino	4	None	81	None	134.5	135–138
2A	*m*-Tolylamino	5	90	73	None	None	89–91
3A	Diphenylamino	6	60	84	[b]	None	114–117
4A	*o*-Anisylamino	7	None	61	None	48–48.5	48–49
5A	Benzylamino	8	None	66	[c]	93.5–94.5	93–95
6A	Ethylene-bisamino	9	59	39	None	174–176	171–175
7A	*p*-Bromophenylamino	9	None	78	None	None	170–173
8A	*p*-Nitrophenylamino	10	90	75	None	182–184	181–184
9A	*p*-Sulfophenylamino	11	40	40	None	None	285 (decompn.)
10A	*p*-Hydroxyphenylamino	12	65	58	None	144.5–146	141–147
11A	*p*-Carbethoxyphenylamino	13	None	51	None	116	110–115
12A	Carboxymethylamino	14	70	..	[d]	98–100	.....
13A	2-Phenylethylamino	15	None	44	[e]	67	63–65
14A	Phenylazophenylamino	16	50	42	None	155–9	154–155
15A	N,N′-Piperazyl bis-	17	None	43	[f]	137	130–134
16A	*m*-Acetylphenylamino	18	69	41	[g]	113.5–114.5	114–116
17A	2-Pyridylamino	19	80	34	[h]	110	112–114
18A	Morpholinyl	20	None	49	[i]	oil	[j]

[a] All melting points are uncorrected. [b] The anhydrous ether method worked better than Kelsey's aqueous method. [c] Alcohol-water was used as the recrystallization solvent instead of benzene used by Jacobs. [d] Oil obtained which was used as such without further purification. [e] Product recrystallized from benzene plus toluene instead of ether. [f] Product recrystallized from alcohol. [g] This product was recrystallized twice from alcohol and water and once from benzene. [h] Carbon tetrachloride instead of petroleum ether used as a recrystallization solvent. [i] For better solubility behavior, chloroform instead of ether was used. [j] Lit. b. p., 130–131°/3 mm.; obs. b. p. 186–190°/18 mm.

treated with either aqueous or hydroalcoholic solutions of L-cysteine at room temperature readily yielded the corresponding sulfides. Sodium bicarbonate was used primarily as the hydrogen halide acceptor. Most of the sulfides generally appeared as white precipitates which were then made soluble by the addition of a 20% solution of sodium hydroxide, filtered, and finally treated either with dilute or concentrated hydrochloric acid to a slightly acid pH to precipitate out the amphoteric compounds. The yields of these sulfides were generally fair and the reaction time involved about two to three hours.



EXPERIMENTAL

Mono- and Di-Substituted Chloroacetamides (Table I).—These intermediates were prepared by either one of two well-established methods. The one, or the nonaqueous method, utilizes two equivalents of the amine for each equivalent of chloroacetyl chloride. The other procedure makes use of about a 20% molar excess of chloroacetyl chloride to the amount of the amine, the reaction being carried out in water plus base.

Most of the amines used as starting material were Eastman chemicals, otherwise they were obtained as follows: 1A and 9A from Baker and Adamson; 3A from Allied Chemical; 4A and 6A from Matheson; 11A from Revson; and 12A from Merck.

L-3-(Mono- and Di-substituted)-carbamylmethylthioalanines Table II).—Compounds 1, 4, 6, 10, 15, and 16 were purified by recrystallization from water. Compounds 9, 12, and 17 were recrystallized from approximately equal parts of acetic acid and water. For compound 2, a 10% solution of ethanol in water was used, while compound 3 required an initial recrystallization with alcohol and water followed by a second recrystallization from approximately equal parts of acetic acid and water. Compound 8 was recrystallized from a solution of three volumes alcohol and two volumes water; number 13 required four volumes water and one volume acetic acid; number 18 required nine volumes methanol and one volume acetic acid.

The procedure for the preparation of L-3-(phenylcarbamylmethylthio)-alanine will serve to typify the procedure employed for the remaining sulfides in this group. The preparation of L-3-phenylazophenylcarbamylmethylthioalanine has, however, also been singled out because it was necessary to depart from this method.

L-3-Phenylcarbamylmethylthioalanine.—A mixture consisting of 6.4 Gm. (0.038 mole) of α-chloroacetanilide, 6.6 Gm. (0.038 mole) of L-cysteine hydrochloride monohydrate,[1] 9.6 Gm. (0.114 mole) of sodium bicarbonate, 80 ml. of water, and 50 ml. of alcohol was vigorously agitated in a flask for three hours. With continued agitation, a 10% solution of sodium hydroxide was added, dropwise, until all but a slight amount of precipitate remained in the flask (pH 9). The chilled filtrate, acidified to

[1] Products from Nutritional Biochemicals and Eastman were used.

TABLE II.—L-3-(MONO- AND DI-SUBSTITUTED)-CARBAMYLMETHYLTHIOALANINES

No.	N	Formula	Yield, %	M. P. °C. (decompn.)	$[\alpha]_D^{25}$ [a]	—Nitrogen, %— Calcd.	Found [c]	—Sulfur, %— Calcd.	Found
1	Phenylamino	$C_{11}H_{14}N_2O_3S$	79.0	199	14.1°	11.02	10.88	12.61	12.90
2	*m*-Tolylamino	$C_{12}H_{16}N_2O_3S$	65.8	195	17.8°	10.44	10.38	11.95	11.70
3	Diphenylamino	$C_{17}H_{18}N_2O_3S$	24.1	173	14.7°	8.48	8.28	9.70	9.73
4	*o*-Anisylamino	$C_{12}H_{16}N_2O_4S$	37.9	150	9.15°	9.86	9.47	11.28	11.40
5	Benzylamino	$C_{12}H_{16}N_2O_3S$	23.6	205	−0.3°	10.44	10.32	11.95	12.08
6	Ethylene-bisamino	$C_{12}H_{22}N_4O_6S_2$	26.3	229	8.7°	14.65	14.40	16.76	16.70
7	*p*-Bromophenylamino	$C_{11}H_{13}BrN_2O_3S$	35.0	209	16.98°	...	[d]	9.62	10.00
8	*p*-Nitrophenylamino	$C_{11}H_{13}N_3O_5S$	32.2	189	[b]	14.04	13.88	10.71	11.00
9	*p*-Sulfophenylamino	$C_{11}H_{14}N_2O_6S$	52.2	255	7.41°	8.38	8.06	19.17	19.45
10	*p*-Hydroxyphenylamino	$C_{11}H_{14}N_2O_4S$	54.2	211	19.65°	10.37	10.29	11.86	11.54
11	*p*-Carbethoxyphenylamino	$C_{14}H_{18}N_2O_5S$	32.6	203	17.80°	8.58	8.55	9.82	10.00
12	Carboxymethylamino	$C_7H_{12}N_2O_5S$	10.0	184	−15.8°	11.86	11.71	13.57	14.25
13	2-Phenylethylamino	$C_{13}H_{18}N_2O_3S$	39.2	198	4.1°	9.92	9.88	11.35	11.41
14	Phenylazophenylamino	$C_{17}H_{18}N_4O_3S$	23.0	206	8.2°	15.63	15.54	8.95	8.85
15	N,N'-Piperazyl bis-	$C_{14}H_{24}N_4O_6S_2$	44.3	195	8.8°	13.72	13.34	15.70	15.40
16	*m*-Acetylphenylamino	$C_{13}H_{16}N_2O_4S$	55.7	159	15.8°	9.45	9.42	10.82	10.80
17	2-Pyridylamino	$C_{10}H_{13}N_3O_3S$	30.3	176	−0.2°	...	[e]	12.56	12.62
18	Morpholinyl	$C_8H_{16}N_2O_4S$	18.0	168	−14.2°	...	[f]	12.91	12.68

[a] The optical rotation values were ascertained at the Sterling-Winthrop Research Institute. A solution of 1% of the compound in two equivalents of 1 *N* sodium hydroxide was used for compounds 1, 2, 3, 5, 6, 7, 10, 11, and 15. A solution of 1% of the compound in one equivalent of 1 *N* sodium hydroxide was used for compounds 4, 13, and 16. For compound 14, 0.5% was dissolved in one equivalent of potassium hydroxide in methanol. For compounds 9, 12, 17, and 18, a 1% solution in water was used. [b] Unstable in alkaline medium. [c] The analytical data were determined at the Sterling-Winthrop Research Institute. [d] Bromine content was also determined. *Anal.*—Calcd.: Br, 23.99. Found: Br, 24.30. [e] Carbon and hydrogen were also determined. *Anal.*—Calcd.: C, 47.05; H, 5.13. Found: C, 46.97; H, 5.23. [f] Carbon and hydrogen were also determined. *Anal.*—Calcd.: C, 43.53; H, 6.50. Found: C, 43.76; H, 6.16.

pH 4 with dilute hydrochloric acid, readily yielded the white product which was recrystallized three times from hot water. The 7.7 Gm. of product thus obtained corresponded to a 79% yield; m. p. 199° (decompn.). For analytical data, see compound 1 in Table II.

L - 3 - Phenylazophenylcarbamylmethylthioalanine.—In a 1-L., three-necked flask provided with a stirrer, water condenser, and heating mantle, was placed 16.6 Gm. (0.061 mole) of *p*-chloroacetylaminoazobenzene, 10.7 Gm. (0.061 mole) of L-cysteine hydrochloride monohydrate, 15.4 Gm. (0.183 mole) of sodium bicarbonate, 130 ml. of water, and 130 ml. of alcohol. Heating at 65° was maintained for one and one-half hours. Continued agitation and addition of dilute hydrochloric acid to pH 5 yielded an orange-brown mass which was collected on a filter and then treated with 250 ml. of alcohol and dilute hydrochloric acid to pH 1. Warming the mixture gradually effected a solution, which was filtered while hot. The chilled filtrate was treated in the same manner with a 10% solution of sodium hydroxide, warming it slightly to effect a solution (pH 8). The latter, filtered rapidly, yielded the product by adjusting the pH to 6. For analysis, a sample was further purified by subjecting it once again to the alternate treatment with acid and base as previously described. The product was successively washed with water, alcohol, and ether. The 5.0 Gm. of dried powder obtained represented a 23% yield; m. p. 206° (decompn.). For analytical data, see compound 14 in Table II.

L-3-Benzhydrylmethylthioalanine.—A mixture of 3.3 Gm. (0.019 mole) of L-cysteine hydrochloride monohydrate, 4.3 Gm. (0.021 mole) of benzhydryl chloride,[1] 2.5 Gm. (0.063 mole) of sodium hydroxide, and 70 ml. of water was stirred for four and one-half hours at about 90°. After cooling, an ether extract removed about 1 ml. of oil from the flask. The product appeared by acidification to pH 4. Recrystallized twice from alcohol and water, the 0.7 Gm. of product corresponded to a 13% yield; m. p. 198° (decompn.) $[\alpha]_D^{25}$ + 13.8 (1% in two equivalents of 1 *N* sodium hydroxide).

Anal.—Calcd. for $C_{18}H_{17}NO_2S$: N, 4.87; S, 11.16. Found: N, 4.88; S, 11.40.

L - 3 - (2 - Hydroxyethylthio) - alanine.—This compound was made from 8.3 Gm. (0.047 mole) of cysteine hydrochloride monohydrate, 5.9 Gm. (0.047 mole) of 2-bromoethanol, 8.6 Gm. (1.03 mole) of sodium bicarbonate, and 60 ml. of water, the mixture stirred for three hours at 40°. The pH was then adjusted to 9 by the addition of a 20%

[1] Matheson's benzhydryl chloride redistilled: b.p. 157–159°/22 mm.

solution of sodium hydroxide. An extraction with ether removed a slight amount of an oil. Concentrated hydrochloric acid was added to pH 4. Reduced pressure distillation removed all of the water, following which the dry cake was extracted twice with 50-ml. portions of boiling absolute methanol. Most of the product crystallized from the chilled filtrate. An additional quantity was obtained by adding an equal volume of anhydrous ether to the mother liquor. Both batches were combined and recrystallized twice from 90% alcohol. Yield, 2.5 Gm. (32%); m.p. 193° (decompn.); $[\alpha]_D^{25}$ − 27.5° (1% in water).

Anal.—Calcd. for $C_5H_{11}NO_3S$: N, 8.48; S, 19.41. Found: N, 8.48; S, 19.78.

L-3-(2-Ethoxyethylthio)-alanine.—This compound was prepared according to the foregoing procedure, except that sodium hydroxide was used in place of sodium bicarbonate. The reaction time was four hours at 40°. The product isolated from the methanol extract was recrystallized twice either from water and methanol or from water and alcohol. Yield, 4.4 Gm. (24.4%) of white solid, m. p. 221° (decompn.) $[\alpha]_D^{25}$ − 22.7° (1% in water).

Anal.—Calcd. for $C_7H_{15}NO_3S$: N, 7.25; S, 16.59. Found: N, 7.15; S, 16.60.

BIOLOGICAL RESULTS[1]

Compounds 1, 2, 5, 6, L-3-benzhydrylthioalanine, and L-3-(2-ethoxyethylthio)-alanine were ineffective against poliomyelitis, rabies, and influenza-type viruses in female Swiss mice. L-3-(2-Hydroxyethylthio)-alanine did show, however, a slight activity against a poliomyelitis virus as well as a slight activity against a *Klebsiella pneumoniae* infection, when given intraperitoneally in aqueous solution twice a day to female Swiss mice. Others prepared in this study were found to be ineffective against *Micrococcus pyogenes* "Swiss" strain, *Klebsiella pneumoniae*, and mycotic infections of *Candida albicans*. Also, most of these which were tested gave negative results as possible antimetabolites for calcium pantothenate, vitamin B_{12}, folic acid, serine, cystine, phenylalanine, tryptophan, and histidine, using various organisms. Compounds 1 and L-3-(2-hydroxyethylthio)-alanine were ineffective as antitubercular agents. Against a *Trichomonas gallinae* infection in hamsters, compounds 7 and 8 were without effect, but L-3-benzhydrylthioalanine was effective for three of the five animals tested, when given into the gluteus muscle in 10% gelatin via a stomach tube twice daily, five days a week for two weeks.

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Potentiation of Preservatives (Parabens) in Pharmaceutical Formulations by Low Concentrations of Propylene Glycol

By P. S. PRICKETT, H. L. MURRAY, and N. H. MERCER

Pharmaceutical formulations similar to simple syrups near neutral pH are difficult to preserve against microbial spoilage, which sometimes require high concentrations of preservatives, e. g., parabens. Since high preservative concentrations injure formulations' palatability, inclusion of an appropriate potentiator would be helpful. Potentiation of parabens by low levels of propylene glycol (PG) was investigated. Four groups, each consisting of three formulations were studied. Levels of PG (0, 2, and 5 per cent) were constant in all groups, but the paraben levels differed for each formulation group with one group (control) containing none. Samples of each formulation were inoculated with bacteria, molds, and yeasts, respectively, then incubated up to six months. The low level paraben group showed strong potentiation by PG. The other two paraben groups demonstrated lesser degrees of potentiation. However, in short-time studies in which the formulations received multiple bacterial inoculations, PG potentiated even the highest concentration of parabens.

LIQUID ORAL preparations comprise one of the largest classes of pharmaceuticals. In common with all pharmaceuticals, the primary problem is stability; secondary, acceptability. Attainment of both objectives is usually required by the pharmaceutical manufacturer, the physician, the retailer, and the patient.

Since the stability of many drugs in solution is dependent on pH, this factor is often rigidly established. Also, most of the acceptable preservatives have decreased action against contaminating organisms at a pH near neutrality. Therefore, for those drugs which require a pH near neutrality, preservation of the formulation usually presents additional problems. The problem of decreased activity can be overcome in some instances by increasing the concentration of the preservative used. This approach, however, introduces problems such as decreased acceptability due to undesirable taste of excess preservative, exceeding the solubility of the preservative during exposure to low temperatures, and increased cost. To overcome these problems, a substance which would potentiate preservatives at near neutral pH's would be very desirable.

The use of propylene glycol was first suggested as a vehicle for medicinal purposes in 1931 (1). Substitution of propylene glycol for alcohol and/or glycerin in thirty-nine N. F. or U. S. P. preparations resulted in formulations which were deemed satisfactory in the majority of cases (2). Factors considered were taste, cost, compatibility, and stability. References to the use of propylene glycol as a solvent or vehicle for liquid pharmaceuticals are not uncommon in the literature. However, a fairly comprehensive survey of the literature over the last ten years revealed only two references to the use of propylene glycol as a potentiator for preservatives (3, 4). In both articles, the authors reported potentiation of a number of preservatives. The levels of propylene glycol tested in one case (3) was 0 and 10 per cent v/v and the other investigator (4) used 10 per cent propylene glycol plus 10 per cent sorbitol.

Since propylene glycol increases the cost and makes flavoring of liquid pharmaceuticals more difficult, it was deemed advisable to determine whether potentiation of a widely accepted preservative (parabens) would also occur using less than 10 per cent propylene glycol. In order to derive this information, this study was set up. Three concentrations (0, 2, and 5 per cent) of propylene glycol with four different levels of preservative were studied.

EXPERIMENTAL

Formulations Studied.—The formulations were 45% sucrose (w/v) syrups of near neutral pH (6.2), a type of formulation difficult to preserve against microbial spoilage. The 12 formulations differed only in their respective contents of the preservative, methyl and propyl parabens, and of propylene glycol (PG), as shown in Table I. By using different combinations, the potentiating effect of 2 and 5% PG on various levels of parabens was determined.

METHODS

Preparation of the Test Cultures.—Each type of inoculation consisted of a mixture of pure cultures to simulate various natural contaminations. The

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TABLE I.—COMPOSITION OF THE FORMULATIONS STUDIED[a]

Ingredients	Formulations											
	Group I			Group II			Group III			Group IV		
	A	B	C	D	E	F	G	H	I	J	K	L
Sucrose, %	45	45	45	45	45	45	45	45	45	45	45	45
Methylparaben	0.18	0.18	0.18	0.13	0.13	0.13	0.1	0.1	0.1	..	..	..
Propylparaben	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	..	..	..
Propylene glycol	..	2.0	5.0	..	2.0	5.0	..	2.0	5.0	..	2.0	5.0
Distilled water	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*	*q. s.*

[a] Adjusted pH to 6.0–6.5 with sodium citrate.

formulations were not diluted significantly by the inocula as they consisted of less than 1 cc. of aqueous suspensions of heavy cell concentrations.

The inocula used for testing the formulations' effect against nonspore forming bacteria were fresh cell suspensions of six test species. These organisms, often found in the upper respiratory tract, were: *Pseudomonas aeruginosa* A.T.C.C. 9027, *Escherichia coli* A.T.C.C. 3226, *Staphylococcus aureus* (albus form) A.T.C.C. 151, *Sarcina lutea* A.T.C.C. 9341, *Micrococcus flavus* A.T.C.C. 10240, and *Neisseria sp.*

The organisms were grown in trypticase soy broth[1] for twenty-four hours at 35°, then transferred to tryptone glucose yeast (TGY) agar[2] slants for twenty-four hours at 35°. Following incubation, the cells were harvested with 0.9% saline and the suspension standardized with a Lumetron photoelectric colorimeter, model 401. Table II lists the per cent light transmissions of the culture suspensions plus their respective plate counts.

TABLE II.—STANDARDIZED CELL SUSPENSIONS

Culture	Per Cent Light Transmission	Plate Count per cc.
0.9% Saline	100	0
Ps. aeruginosa	55	4.9×10^8
E. coli	79	3.5×10^8
Staph. aureus (albus form)	54	2.25×10^8
S. lutea	50	3.5×10^8
M. flavus	23	9.4×10^8
N. sp.	53	3.5×10^8

Each standardized suspension was diluted with 0.9% saline to produce approximately 10^8 organisms per cc., then pooled to obtain the stock inoculum, which was then further diluted to procure the desired level of inoculation.

The mold inoculum consisted of two penicillium species and one each of *Oospora*, *Alternaria*, and *Aspergillus*. The cultures were grown on malt agar[2] slants for five days at 25°, then harvested with the aid of a loop and about five cc. of sterile buffered water. The harvest from each slant was pooled in a bottle (*q. s.* 100 cc. with sterile buffered water) containing glass beads and shaken thoroughly. The pool was then filtered through sterile glass wool to separate the spores from the mycelium. The filtrate (containing the spores) was kept at 8° until it was used. The number of viable mold spores in the suspension, determined by plate count, averaged 6×10^6 per cc. The suspension was diluted to give the desired level of inoculation.

[1] Baltimore Biological Laboratory, Baltimore, Md.
[2] Difco Laboratories, Inc., Detroit, Mich.

Five yeast cultures were employed in the pooled yeast inoculum. One of the cultures was *Saccharomyces cerevisiae* A.T.C.C. 9763, while the other four were *Saccharomyces spp.* that had been isolated from various spoiled products. The cultures were grown on malt agar slants at 25° for ninety-six hours, at which time they were harvested with 5 cc. of buffered sterile water per slant. The pooled harvest from the five slants when *q. s.* 100 cc. yielded approximately 5×10^7 cells per cc. This stock suspension was further diluted to obtain the desired level of inoculation.

Four species of Bacillus (*B. cereus* A.T.C.C. 7064; *B. subtilis* A.T.C.C. 9524; *B. brevis* A.T.C.C. 10027; *B. megatherium* strain 234, A.T.C.C. 8245) were used to provide the inoculum of bacterial spores. The spore suspension was prepared by harvesting the growth of each species with distilled buffered water from a slant of TGY that had been incubated for four days at 30°. The cells were pooled, centrifuged, washed three times, and resuspended in sterile water (50 cc.). This suspension was then heated for twenty minutes at 80° and stored at 8° until ready for use. As the viable count of this stock suspension was 2×10^8 per cc., no further dilution was needed for the desired inoculum level.

The predetermined levels of inoculation of the formulations with each type of inoculum is shown as part of Tables III–VII.

Materials.—Noninoculated samples (30-cc. bottles) of all formulations were included as controls in all tests. Bottles that were inoculated during the microbial stability testing contained 30 cc. while those used in the bacterial capacity test held 50 cc.

Malt agar was used in plating samples inoculated with yeasts or molds, whereas TGY was employed in plating those bottles inoculated with bacteria.

Microbial Stability.—Samples of each formulation were inoculated respectively with cultures of nonspore forming bacteria, molds, spores, yeasts, and bacterial spores. Platings were made of the inocula to confirm the number of organisms added to the samples, as they were inoculated to contain a predetermined, initial level of viable cells per cc. of formulation.

Immediately after inoculation each set of bottles was plated to determine its viable microbial population at the "zero hour," and then equal numbers of bottles were incubated at 25 and 35°, respectively. At periodic intervals the bottles were assayed for their respective microbial populations.

Bacterial Capacity Test.—Samples of each formulation were warmed to 35°, then inoculated to a predetermined level per cc. of formulation with the simulated nasopharyngeal flora, immediately plated for the zero hour counts, and incubated at 35°. After six hours of incubation, the surviving bacterial populations were determined. During the next

TABLE III.—ANTIBACTERIAL (NONSPORE FORMERS) ACTIVITY OF COMBINATIONS OF METHYL AND PROPYL PARABENS AS AFFECTED BY PROPYLENE GLYCOL (PG)

Formulations[a]	Paraben Concn., % Methyl	Paraben Concn., % Propyl	Prop. Glycol Concn., %	Viable Bacterial Counts/cc.[b] Formulation, After Inoculation and Incubation at 25°: 0 hr.	1 wk.	2 wk.
Group I						
A	0.18	0.02	0	18	0	0
B	0.18	0.02	2	12	0	0
C	0.18	0.02	5	18	0	0
Group II						
D	0.13	0.02	0	22	0	0
E	0.13	0.02	2	11	0	0
F	0.13	0.02	5	19	0	0
Group III						
G	0.10	0.02	0	22	0	0
H	0.10	0.02	2	30	0	0
I	0.10	0.02	5	19	0	0
Group IV						
J	0	0	0	28	14[c]	5[c]
K	0	0	2	26	8[c]	0[c]
L	0	0	5	27	10[c]	5[c]

[a] Bacterial inoculum = 30,000/cc. formulation. [b] Factor for counts = ×1,000. [c] Molds also on plates; grew from "natural" contaminants in formulations.

TABLE IV.—ANTIMOLD ACTIVITY OF COMBINATIONS OF METHYL AND PROPYL PARABENS AS AFFECTED BY PROPYLENE GLYCOL (PG)

Formulations[a]	PG Concn., %	Viable Mold Counts/cc.[b] Formulation After Inoculation and Incubation at 25°: 0 hr.	1 wk.	2 wk.	3 wk.	4 wk.	6 wk.	2 mo.	3 mo.	4 mo.	5 mo.	6 mo.
Group I												
A	0	66	0	0.1	0	0	0	0	0	0	0.9	0.2
B	2.0	69	0.2	0	0	0	0	0	0	0	0	0
C	5.0	76	0.2	0	0	0	0	0	0	0	0	0
Group II												
D	0	92	0	1.0	0	0	0	0	0	0	0	0
E	2.0	83	0.3	0	0	0	0	0	0.2	0	0.1	0.5
F	5.0	78	0.3	0	0	0	0	0	0	0	0	0
Group III												
G	0	94	1.1	0.2	0	0	0.1	0.1	0	0.5	1.0	2.2
H	2.0	98	0	0	0	0	0	0	0	0	0	0
I	5.0	89	0.9	0	0	0	0	0	0	0	0	0
Group IV												
J	0	69	38	37	64	44	...	...	...	...	...	...
K	2.0	43	42	20	26	50	...	...	...	...	...	...
L	5.0	65	42	29	37	65	...	...	...	...	...	...

[a] Mold inoculum = 95,000/cc. of formulation. [b] Factor for counts = ×1,000.

TABLE V.—ANTIYEAST ACTIVITY OF COMBINATIONS OF METHYL AND PROPYL PARABENS AS AFFECTED BY PROPYLENE GLYCOL (PG)

Formulations[a]	PG Concn., %	Viable Yeast Counts/cc.[b] Formulation After Inoculation and Incubation at 25°: 0 hr.	1 wk.	2 wk.	3 wk.	4 wk.	6 wk.	2 mo.	3 mo.	4 mo.	5 mo.	6 mo.
Group I												
A	0	48	0.7	3.0	4.0	0.9	0.6	2.0	1.0	2.0	0.2	0.4
B	2.0	50	0.2	0	0	0	0	0	0	0	0	0
C	5.0	31	0	0	0	0	0	0.8	0	0	0	0
Group II												
D	0	51	5.0	8.0	2.0	1.0	0.7	5.0	3.0	0	1.0	1.2
E	2.0	32	0.3	0	0	0.4	0	5.0	0	0	3.0	1.2
F	5.0	72	1.8	0	0	0	0	0	0	0	0	0
Group III												
G	0	38	9.0	0.4	1.0	6.0	7.0	3.3	4.0	7.0	9.0	3.1
H	2.0	72	7.0	8.0	5.0	3.0	2.0	2.0	1.2	0.2	0.3	0.5
I	5.0	37	2.0	0	0	0	0	0	0	0	0	0
Group IV												
J	0	67	237	217	348[c]	[d]	...	...	...	...	...	...
K	2.0	66	207	230[c]	278[c]	[d]	...	...	...	...	...	...
L	5.0	62	99	84	161[c]	[d]	...	...	...	...	...	...

[a] Yeast inoculum = 85,000/cc. of formulation. [b] Factor for counts = ×1,000. [c] Molds, "natural" contaminants, also on plate. [d] Molds overgrew plate.

TABLE VI.—ANTISPORE (BACTERIAL) ACTIVITY OF COMBINATIONS OF METHYL AND PROPYL PARABENS AS AFFECTED BY PROPYLENE GLYCOL (PG)

Formulations[a]	PG Concn., %	Viable Bacterial Spore Counts/cc.[b] Formulation After Inoculation and Incubation at 25° 0 hr.	1 wk.	1 mo.	2 mo.	3 mo.
Group I						
A	0	17	24	27	26	21
B	2.0	14	17	28	14	25
C	5.0	20	20	20	26	14
Group II						
D	0	18	13	27	27	28
E	2.0	26	28	24	18	11
F	5.0	28	27	17	18	14
Group III						
G	0	17	25	14	14	11
H	2.0	26	18	24	20	11
I	5.0	18	11	22	21	17
Group IV						
J	0	..	..	..	..	..
K	2.0	..	..	..	..	..
L	5.0	..	..	..	..	..

[a] Spore inoculum = 30,000/cc. of formulation. [b] Factor for counts = × 1,000.

several days the same bottles were reinoculated the second and third times to the desired levels with fresh preparations of the same flora. After each inoculation of the bottles, viable bacterial levels were determined at the same time intervals. Each cell suspension that was used as the inoculum was plated to confirm its viable population at the time the bottles were inoculated.

RESULTS

Controls.—In the paraben-containing formulations of Groups I, II, and III, none of the control samples (noninoculated) showed evidence of viable cells. However, in the paraben-free control formulations of Group IV, the natural contaminants (bacteria, molds, and yeasts already present in the formulations) grew to significant levels within approximately a week. It was found that propylene glycol *per se* had a mild inhibitory effect in that the higher the PG concentration the slower the growth of the natural contaminants.

Microbial Stability.—*Bacteria (Nonspore Forming Species).*—The results in Table III show that all of the formulations in groups I, II, and III were able to kill the inoculated, nonspore forming bacteria within a week. The fact that these inoculants were killed by the group III formulations with the lowest concentration of parabens indicates that these low concentrations are effective against vegetative bacterial cells. In contrast, the paraben-free formulations in Group IV not only failed to kill all of the inoculants within two weeks but even permitted the "natural" mold contimants in the formulations to develop.

Molds.—In groups I, II, and III the influence of two concentrations of PG on various levels of parabens is shown by the mold counts of the inoculated bottles listed in Table IV.

The counts show that as the concentration of the parabens is reduced, i. e., from the level in group I to that in group III, the potentiating effect of PG becomes more evident.

The skips observed in Table IV are rather a common occurrence in this type of test. The reasons have not been worked out completely, but presumably they involve "acclimitization" of the inoculated molds to the inhibitory factors of the formulations.

The formulations containing PG alone (group IV) had a mild inhibitory effect against the inoculated molds.

Yeasts.—Table V shows the potentiating effect of PG on the parabens' activity against the inoculated yeasts. Although the patterns of potentiation are similar to those found in the mold-inoculated formulations, certain significant differences were observed. To illustrate, 5% PG (formulation I,

TABLE VII.—CAPACITY OF PARABENS TO KILL REPEATED INOCULATIONS OF NONSPORE FORMING BACTERIA AS AFFECTED BY PROPYLENE GLYCOL (PG)

Formulations[a]	PG Concn., %	1st Inoculation[c] Inoculation Level[e]	0 hr.[d]	6 hr.[d]	2nd Inoculation[b] Inoculation Level[e]	0 hr.[d]	6 hr.[d]	3rd Inoculation[c] Inoculation Level[e]	0 hr.[d]	6 hr.[d]	48 hr.[f]
Group I											
A	0	200	135	0	500	122	0	300	130	65	6.3
B	2.0	200	138	0	500	344	0	300	89	38	0
C	5.0	200	110	0	500	259	0	300	45	4	0
Group II											
D	0	200	180	35	500	255	129	300	350	...	60
E	2.0	200	160	8	500	250	35	300	142	87	1.5
F	5.0	200	125	0	500	181	6	300	78	24	0
Group III											
G	0	200	132	19	500	355	62	300	291	82	24
H	2.0	200	133	18	500	350	56	300	263	79	14
I	5.0	200	107	15	500	305	19	300	237	31	1
Group IV											
J	0	200	165	90	500	550	310	300	410	340	...
K	2.0	200	160	122	500	580	330	300	380	150	96
L	5.0	200	168	119	500	560	350	300	480	360	120

[a] Factor for bacterial counts = ×1,000.
[b] Second inoculation made two days after the first inoculation.
[c] Third Inoculation made seven days after the second inoculation.
[d] Bacterial counts per cc. of formulation at the designated time intervals after the respective inoculations.
[e] Inoculation level is the predetermined amount of inoculum added to give indicated number of viable bacteria per cc. of formulation. Fresh inocula were prepared for each of the three inoculations.
[f] The bacterial count made forty-eight hours after the last of the inoculations was to allow ample time for the preservatives to exert their effect.

group III, Table V) was required to potentiate the lowest level of parabens to eliminate viable yeasts, whereas only 2% PG was required for similar potentiation to kill the molds (H, group III, Table IV). Furthermore, propylene glycol alone, (K and L, group IV, Table V) permitted yeasts to multiply, whereas the molds were unable to do so under the same conditions (K and L, Table IV).

Bacterial Spores.—The results with spores are given in Table VI. The results obtained with the formulations in groups I, II, and III are sufficient to show the relative ineffectiveness of parabens, with and without the potentiator, on bacterial spores. Thus, since we have observed that spores *per se* do not cause spoilage, even after long storage, this phase was terminated after three months.

Bacterial Capacity Test.—Since certain types of pharmaceutical formulations are subjected to repeated bacterial contamination by the user, these types of formulations should have sufficient preservative capacity to protect themselves against microbial spoilage under such conditions. Thus, it was desirable to study whether PG would maintain its potentiating effect on the parabens as long as they maintained their activity in the face of repeated, heavy bacterial inoculations.

The results in Table VII show that PG did maintain its potentiating effect on the parabens even after multiple inoculations totaling a million vegetative cells per cc. of formulation. This is presumably a heavier total contamination than would be anticipated during average use. It is to be noted that each of the multiple inoculations consisted of the same combination of various nonspore forming species simulating the nasopharyngeal flora.

The paraben-free formulations in group IV showed that PG alone, in the concentrations tested, had little significant effect on the growth of the inoculated bacteria. In these formulations the zero hour counts following the second and third inoculations were higer than that of the inoculum. This was due to viable cells remaining from the previous inoculation.

DISCUSSION

This study, as well as previous ones, demonstrated the adequate preservative (antimicrobial) effect of the higher concentrations of the parabens used in this investigation. In this respect, our results confirm those of Neidig and Burrell (5). As anticipated, it was at the lower levels of the combinations of the parabens (approximating their minimal preservative concentration) that the potentiating effect of propylene glycol was most evident. (Tables IV, V, and VII.)

Moreover, this effect was shown by significantly lower concentrations of PG than was studied by Neipp (3). At a 10% level he found that PG has a potentiating effect on various antimicrobials, such as antibiotics, quaternary ammonium compounds, and salicylanilides; parabens were not included in his study.

Barr and Tice (4) found that the addition of 10% PG to sorbitol solutions produced an inhibitory effect that was not due entirely to osmosis. They concluded the effect of PG is probably related to its chemical structure. Our results also suggest that the potentiating effect of low levels of PG is not dependent solely on its osmotic effect. The potentiating effect of PG was evident at the two incubation temperatures of 25 and 35°. Since the results obtained at both temperatures were essentially the same, and since 25° is the optimum temperature for the mold and yeast inocula, to simplify the tables, only the data obtained at 25° are presented.

Only one form of potential spoilage organisms, the bacterial spores (Table VI), failed to respond to the potentiating action of PG. This lack of effect is not surprising in view of the relatively high resistance of spores to many chemicals as well as to heat. However, when the spores germinate, the resultant vegetative cells would be subject to the potentiating action of PG on the preservative as is seen in Table VII. On this basis, PG would be expected to potentiate the preservative action of the parabens against potential spoilage due to bacterial spores.

CONCLUSIONS

1. Propylene glycol, as the only preservative agent in these formulations, did not demonstrate significant antimicrobial activity at low concentrations of 2 and 5 per cent; thus, at these levels it is an inadequate preservative.

2. Although none of the three levels of the paraben combinations on test permitted visible spoilage of the formulations, viable cell counts showed that significant numbers of the inoculants remained viable. In contrast, the low levels of PG enhanced the antimicrobial activity of the parabens to the point that even at the lowest concentration of parabens, practically all of the inoculants (except for bacterial spores) were destroyed.

3. Even though PG does not potentiate the parabens against bacterial spores *per se*, it does potentiate against the vegetative cells resulting from the germination of the spores.

4. The potentiating effect of PG on the antimicrobial activity of the parabens in liquid pharmaceutical preparations may be applied practically: (*a*) to increase the preservative action in formulations difficult to protect against microbial spoilage, and (*b*) to reduce the concentration of parabens thereby improving the flavor and consumer acceptance.

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Structural Studies on Related Argemone Alkaloids

By LEMONT B. KIER† and TAITO O. SOINE

The alkaloids argemonine, $C_{16}H_{10}(OCH_3)_4NCH_3$; norargemonine, $C_{16}H_{10}(OCH_3)_3(OH)NCH_3$; and bisnorargemonine, $C_{16}H_{10}(OCH_3)_2(OH)_2NCH_3$ are considered to be tetrahydroisoquinolines based on oxidation products and the inability to hydrogenate argemonine further. The benzoylation reaction of bisnorargemonine is typical of aporphine alkaloids. Oxidation studies indicate that the hydroxyl group of norargemonine is in ring D while the two hydroxyl groups of bisnorargemonine are in rings A and D. The Hofmann degradation of argemonine yielded successively a vinyl dihydrophenanthrene methine and an unexpected pyran. Oxidation of the pyran gave an expected tricarboxylic acid. Hydrogenation of the methine followed by the Hofmann degradation gave the expected ethyl phenanthrene. It is tentatively proposed that argemonine has a 6,7 placement of methoxyl groups in ring A and of the three possible vicinal placements of methoxyl groups in ring D, the 2,3 positions appear to be the most likely.

PREVIOUS STUDIES on argemone species in these laboratories (1–3) have resulted in the isolation of three new alkaloids named argemonine, norargemonine, and bisnorargemonine.[1] The original investigation (1) reported the isolation of argemonine, $C_{21}H_{25}NO_4$, and norargemonine, $C_{20}H_{23}NO_4$, from *A. hispida*. A later communication (2) showed that norargemonine yielded argemonine when methylated. A subsequent report (3) indicated that bisnorargemonine, $C_{19}H_{21}NO_4$, isolated from *A. munita* subsp. *rotundata* collected in Utah, likewise could be converted to argemonine by methylation. Thus, close relationship of the three alkaloids was established as illustrated by the following comparison:

argemonine	$= C_{16}H_{10}(OCH_3)_4(NCH_3)$
norargemonine	$= C_{15}H_{10}(OCH_3)_3(NCH_3)(OH)$
bisnorargemonine	$= C_{16}H_{10}(OCH_3)_2(NCH_3)(OH)_2$

In view of the possibility that the earlier work (1) may well have been carried out on *A. munita* subsp. *rotundata* from Nevada rather than on *A. hispida* (3) it was not surprising that a close relationship existed between these alkaloids.

The structural problem, therefore, resolved itself primarily into a study of the structure of argemonine and, specifically, of the $C_{16}H_{10}$ residue which presumably represents the composition of the basic ring system. Degradative experiments have been employed to elucidate certain features of this nucleus. Schermerhorn and Soine (2) had obtained *m*-hemipinic acid (4,5-dimethoxyphthalic acid) by permanganate oxidation of argemonine which, together with other findings, had suggested an isoquinoline nucleus. This hypothesis was reasonable because virtually all alkaloids obtained from papaveraceous plants are derivable from or related to isoquinoline. Considering the available data at that time, these authors concluded that a 1-benzyltetrahydroisoquinoline structure was unlikely because, in order to satisfy the analytical data, such a structure would have to contain a double bond in addition to the two aromatic nuclei inferred to be present. In order to account for the optical activity of the base, the double bond could be only in the 3,4-position and should be easily reduced or brominated. Neither of these treatments was successful. Certain findings in the present work also tend to disprove the 1-benzyltetrahydroisoquinoline concept of the structure. Bis-benzylisoquinolines, on the basis of molecular weight determinations, obviously do not enter into the structural argument. The fact that an N-methyl group is found in argemonine eliminates the tetrahydroberberine group from consideration. Without too much evidence, Schermerhorn and Soine suggested that a structure embodying a fused four-ring system containing a tetrahydroisoquinoline and benzene nucleus would be the most likely and would meet the analytical and other requirements. The present work helps to place this hypothesis on a firmer basis. The most likely system, and one not uncommon in Papaveraceae, is the aporphine class of alkaloids typified by the well-known glaucine:

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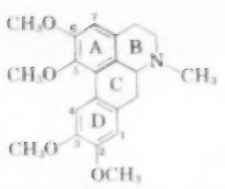
[1] Bisnorargemonine was unfortunately named "rotundine" in the earlier paper (3) describing its isolation and characterization. It is recommended that the name "bisnorargemonine" be adopted to avoid confusion with the $C_{19}H_{21}NO_3$ alkaloid named rotundine which was isolated by Kondo and Matsuno (4) from *Stephania rotunda* Loureio. Its structure has been under study more recently by Sugasawa and co-workers (5, 6) who kindly called our attention to the discrepancy in nomenclature.



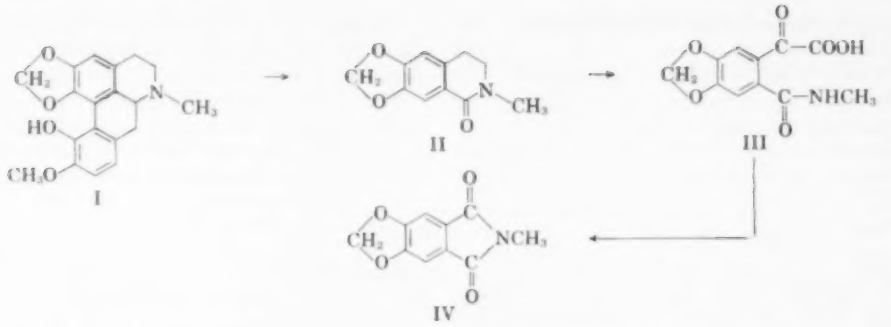
Glaucine

Other members of this class are bases such as corytuberine, corydine, boldine, bulbocapnine, dicentrine, and several others. Indeed, glaucine is a tetramethoxyaporphine isomeric with argemonine and, from the outset, the possibility that argemonine was a member of this group was strongly suspected. That it was not *l*-glaucine was evident from differences in magnitude of optical rotation, melting point, and derivatives. Another possibility, namely dimethylcorytuberine (unknown in nature), was eliminated on the basis of a comparison of the respective tetraphenolic hydrochlorides obtained by demethylation of bisnorargemonine and corydine. In addition, the methine bases derived from argemonine and methylated corydine were quite dissimilar.

Manske (7) proposes that vigorous benzoylation of an aporphine alkaloid will esterify all phenolic hydroxyl groups and, in addition, will effect scission of the nitrogen-containing ring leaving a neutral compound which has lost the typical alkaloidal characteristics. When treated in this manner, bisnorargemonine yielded a material which, although it could not be purified for analysis, was insoluble in both acid and base and failed to give the usual alkaloidal tests. The ultraviolet absorption curve for argemonine corresponded nicely in shape and positions of maxima and minima to that considered typical for aporphines but the intensity of absorption, due to the biphenyl chromophore, was approximately one-half that to be expected. This low intensity of absorption is approximately that to be expected from compounds of the dihydrothebaine type where the chromophore consists of a benzene ring and a conjugated diene structure not conjugated with the benzene ring (8). Likewise, the location of the absorption maximum is approximately that to be expected. However, this type of structure is excluded on the basis of the resistance of argemonine to hydrogenation. For the present, the low intensity of absorption remains unexplained and, indeed, may vitiate some of the following arguments for the aporphine type structure.

Although oxidation of argemonine with potassium permanganate yielded *m*-hemipinic acid (2), oxidation with manganese dioxide in sulfuric acid gave the same acid together with its N-methylimide. The occurrence of the imide as a product of the latter oxidation confirmed the belief that the nitrogen-containing portion of the molecule was a tetrahydroisoquinoline. If argemonine, as seems quite possible, is indeed an aporphine alkaloid, the finding of *m*-hemipinic acid as an oxidation fragment is only explicable by assuming the tetrahydroisoquinoline ring system to be its source. The glaucine arrangement of the two methoxyls on ring D, which has been excluded as a possibility, would be the only other possible source of *m*-hemipinic acid. It is to be noted that the positions of the two methoxyls on ring A have not been unequivocally established by these oxidation products because the arguments concerning decarboxylation of an intermediate tricarboxylic acid could apply equally well to both possible placements of vicinal methoxyls (i. e., 5,6 or 6,7). All attempts to obtain 4,5-dimethoxy-1,2,3-benzene tricarboxylic acid which would be expected as an oxidation fragment of the more biogenetically acceptable 5,6-placement have failed. However, biogenetic considerations may not be sufficient to completely exclude the alternate 6,7-placement in view of the recent findings on the alkaloid crebanine (9). Although the occurrence of the imide as a product of the above oxidation is unusual, it is possible to offer a reasonable explanation. Spath and his co-workers (10) have demonstrated that bulbocapnine (I) yields oxyhydrastinine (II) upon mild permanganate oxidation.

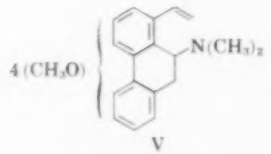
It is to be noted that the carboxyl group, normally expected in the 8-position of the above oxidation product is missing, presumably as a result of decarboxylation. Alkaline permanganate is known (11) to convert (II) to hydrastinic acid (III) which, in turn, is easily oxidized with dilute nitric acid to hydrastic acid methyl imide (IV). A similar sequence of events in the oxidation of argemonine to yield N-methyl-*m*-hemipinimide is not unreasonable. The potassium permanganate oxidation of norargemonine also gave *m*-hemipinic acid. It was concluded, on the basis of the findings with argemonine, that two of the three methoxyls in norargemonine are located on the A ring of the molecule and, therefore, the single phenolic hydroxyl group must be on ring D. Oxidation of bisnorargemonine with either potassium permanganate or manganese dioxide failed to yield significant quantities of any isolable product. From this fact it was suspected that the two phenolic hydroxyls in bisnorargemonine were distributed between rings A and D, and that each labilized its ring to oxidation so that the oxidation fragments



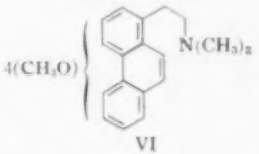
I II III IV

would be small in quantity and probably unrecognizable. In an effort to further locate the two phenolic hydroxyls in bisnorargemonine, the alkaloid was treated with diazoethane. The product could not be readily purified to give a crystalline product but gave every indication of being diethylbisnorargemonine based upon its insolubility in sodium hydroxide solution, solubility in dilute acid, and its positive response to alkaloidal reagents as well as formation of an amorphous picrate. This crude ethylated alkaloid was then oxidized with manganese dioxide to yield 4-ethoxy-5-methoxy-N-methylphthalimide and the corresponding free acid. These products confirmed the belief that the two phenolic hydroxyl groups in bisnorargemonine are distributed between the two aromatic rings.

The classical Hofmann degradation was undertaken in an attempt to obtain recognizable fragments which could be related to the molecule as a whole. The first step of the degradation produced a noncrystalline methine base. Its formula, $C_{22}H_{27}NO_4$, deduced from an analysis of derivatives, agrees with the expected formula. If argemonine is an aporphine alkaloid, there are two possible compounds that can be formed by the first step of the Hofmann degradation (V and VI):



V



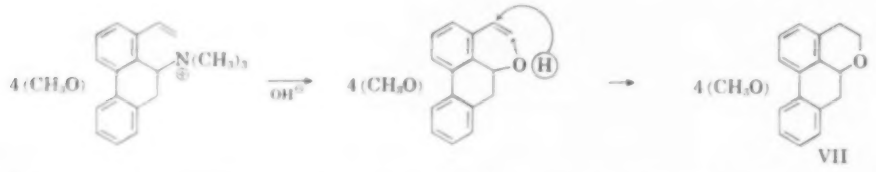
VI

In compound (V), the asymmetric carbon atom adjacent to the nitrogen atom would confer optical activity on the molecule, just as it does in the original alkaloid. In compound (VI), the activity would be destroyed by the formation of the phenanthrene nucleus. The compound actually obtained was optically active and, therefore, it was concluded that the methine base was (V). Infrared analysis of the methine failed to show the expected vinyl group absorption peaks, although the methine hydrochloride showed peaks of a lower intensity than would ordinarily be expected. Both the methine base and its hydrochloride indicated unsaturation by decolorizing a bromine solution without evolution of hydrogen bromide.

To further clarify the matter, the methine base was catalytically hydrogenated as its hydrochloride salt. The saturated base hydrochloride no longer decolorized bromine solution and possessed a different melting point than the unsaturated methine base hydrochloride. Analyses of the hydrogenated methine base gave results which agreed with the $C_{22}H_{29}NO_4$ formula to be expected from hydrogenation of one double bond. The hydrochloride of the dihydromethine base was found to be optically active which corroborates the suggestion that the original methine is (V) inasmuch as only (V) was optically active before hydrogenation and would be expected to be still optically active after saturation of the vinyl group.

The second step of the Hofmann degradation yielded the expected trimethylamine which confirmed the assumption of a tertiary nitrogen with one N-methyl group. However, the nitrogen-free compound did not turn out to be the expected vinyl phenanthrene which is normally obtained from aporphine alkaloids. Instead, the product analyzed for the inclusion of the elements of water in the expected formula. The absence of a hydroxyl absorption peak in the infrared spectrum indicated that the elements of water were not simply solvent of crystallization

nor incorporated as an alcoholic function. The infrared spectrum showed no trace of carbonyl absorption and, therefore, it was concluded that the extra two hydrogens and one oxygen were bound into an ether structure. A possible consideration was the structure (VII) resulting from the following mechanism:



VII

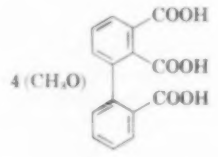
This compound exhibited instability in the presence of water by partially decomposing and showing absorption in the hydroxyl region under infrared analysis. It was optically active and, significantly, the direction of rotation had changed from the original alkaloid.

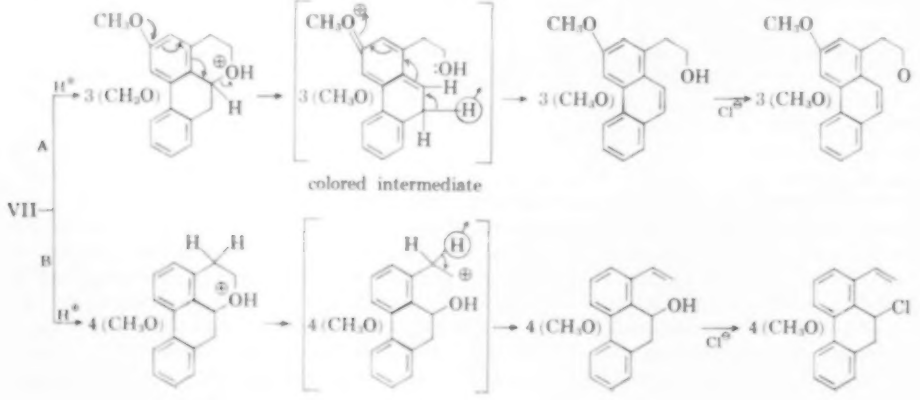
In an effort to further elucidate the character of this ether, it was treated with hydrochloric acid. The reaction was immediate yielding a compound analyzing for $C_{20}H_{21}ClO_4$. It was assumed that the ether linkage had been opened and, of the two possible placements of the chlorine as shown in the two routes (A and B) given below, route A was believed to have taken place. This belief was based on the facts that the compound did not analyze for C-methyl and showed no infrared absorption assignable to a vinyl group. In addition, a transient purple color developed during the reaction which would be explicable on the basis of route A only developing a colored intermediate. The placement of the methoxyl group in route A is unequivocal because the vicinal placement of methoxyls demands that this position be common to both possible placements.

The role of the vinyl group in the formation of the anomalous cyclic ether herein postulated was demonstrated by a Hofmann degradation carried out on the dihydromethine base methiodide which resulted in the anticipated normal dihydro nitrogen-free compound without the inclusion of the elements of water. This compound, as expected, did not show vinyl absorption in the infrared spectrum.

It must be admitted that there is no precedent for this type of ether formation occurring as a consequence of the degradation of any known aporphine alkaloids. However, the evidence seems to indicate some such mechanism to explain this unusual finding.

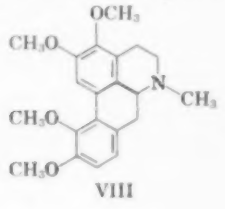
Vigorous permanganate oxidation of the nitrogen-free ether yielded a tribasic acid with concomitant loss of one carbon atom and corresponding to the formula $C_{19}H_{18}O_{10}$. If the three carboxyl groups and four methoxyl groups are each replaced with hydrogen, the molecular formula $C_{12}H_{10}$ arises which, based upon its precursor, can only be biphenyl. The structure of the tribasic acid, therefore, would be:



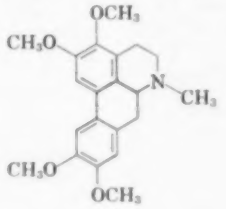


The phthalic acid arrangement of carboxyl groups was indicated by a positive fluorescein test as well as the liberation of a molecule of water at the melting point, presumably during formation of the anhydride. All attempts to decarboxylate the compound resulted in the removal of only one carboxyl group.

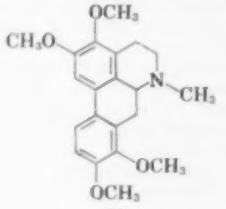
In assessing the accumulated evidence it is apparent that the glaucine and dimethylcorytuberine formulas are definitely excluded as possibilities. Earlier in the course of this study the possibility of the nonbiogenetic arrangement of methoxyl groups in positions 1,2 seemed attractive in view of the work on stephanine and crebanine indicating the acceptability of such a placement. Tomita, however, has reported the synthesis of *dl*-1,2,5,6-tetramethoxyaporphine (12) and given some data on its ultraviolet absorption spectrum. Although there is fair agreement in so far as the shapes of the curves are concerned, the log ε values indicate that argemonine absorbs with about one-half the intensity of the synthetic *dl*-compound (i. e., log ε = 4.35 *vs.* log ε = 4.01 at the maximum). If this possibility is excluded and, if an aporphine structure is assigned to argemonine, there are only three possibilities that can be advanced providing vicinal placement of the methoxyl groups is accepted in ring D. These possibilities are:



VIII



IX



X

On the basis that oxidation procedures have not yet shown any evidence of the presence of *o*-hemipinic acid it would seem that structure (IX) is the most promising. The fact that the two methoxyl groups in ring A do not agree with known biogenetic concepts can be dismissed with the argument that the same was true in the case of crebanine (and stephanine) until the nonbiogenetic placement was proved by synthesis as well as degradation.

There is no question that additional work needs to be carried out on these alkaloids and, indeed, such work is in progress and will be the subject of a future report. It is emphasized that the structural assignments made as a result of the preceding researches are tentative and undoubtedly will need revision as additional evidence is accumulated.

EXPERIMENTAL[2]

Demethylation of Corydine Hydrochloride.—A melting point check on the identity of the corydine hydrochloride showed m. p. 245–255° as contrasted to 258° in the literature. The product (Eastman Kodak white label) was not further identified.

One gram of corydine hydrochloride, 6 Gm. of anhydrous aluminum chloride, and 5 ml. of dry xylene were heated on a wax bath at 140–150° for twenty minutes, the temperature then being raised to 180° for five minutes. The dark colored solution was allowed to cool and ice and concentrated hydrochloric acid added to decompose the complex. A dark green precipitate formed. Upon testing, this dark material was found to have phenolic (ferric chloride test—dark green) and basic (Mayer's test on the difficultly soluble hydrochloride) properties. The mixture was then diluted to twice its volume with water and steam distilled to remove any xylene present. It then appeared that most of the solid material had gone into solution except for a small amount of black oily substance adhering to the sides of the flask. This material was filtered off and the filtrate concentrated under reduced pressure until crystallization occurred. About 200 mg. of product was obtained. Upon further concentration another 460 mg. was obtained. This material, although crystalline, was darker in color than the first. First crop, m. p. 253–258° (decompn.), Thomas-Hoover apparatus, capillary; second crop, m. p. 254–256°. A portion of the second crop was recrystallized from dilute hydrochloric acid to yield crystals, m. p. 256–260° (decompn.). The remainder of the second crop was then also recrystallized, m. p. 259–262°. It was noted as the recrystallizations were carried out that the crystals assumed a dark purple color which was superficial because the interior was still almost white.

Anal.—Calcd. for $C_{17}H_{17}NO_4$: C, 60.80; H, 5.40; OCH_3, 8.84 (one). Found: C, 60.08; H, 5.51; OCH_3, 5.17 (probably due to cleavage of $N—CH_3$).

Demethylation of Bisnorargemonine.—A 0.745-Gm. sample of bisnorargemonine was used. The same procedure for the demethylation of corydine hydrochloride was followed through the point of the decomposition of the complex. At this point the solution was allowed to stand overnight and then the dark green colored precipitate was diluted with enough water to get it into solution at the boiling point (about 250 ml. of water). This was filtered hot through a wetted filter paper to give a clear, slightly yellow colored solution. This was

[2] All melting points were taken on the Kofler apparatus unless otherwise stated.

concentrated under an air stream to one-fourth its volume. Upon standing, the hydrochloride crystallized in the form of white hexahedra, m. p. 225° (and up, indefinite). After two additional recrystallizations from dilute hydrochloric acid a m. p. of 209–213° was obtained when taken very slowly.

Anal.—Calcd. for $C_{11}H_{11}NO_4 \cdot H_2O$: C, 57.71; H, 5.70; OCH_3, 8.84 (one). Found: C, 57.83; H, 5.70; OCH_3, 2.6 (probably due to cleavage of $N\text{-}CH_3$).

Benzoylation of Bisnorargemonine.—A 1-Gm. sample of bisnorargemonine was refluxed with 5 Gm. of benzoyl chloride for thirty minutes. The reaction mixture was then cooled and absolute ether added until a tan precipitate ceased forming. The precipitate was filtered, dried, and recrystallized from petroleum ether to give a microcrystalline product, m. p. 112–116°. The material could not be further purified for analysis but was found to be insoluble in dilute acid and base. An aqueous-alcoholic solution gave no alkaloidal tests.

Manganese Dioxide Oxidation of Argemonine.—One gram of argemonine in 100 ml. of 2.5% sulfuric acid was refluxed with 1 Gm. of manganese dioxide for five hours. The reaction mixture was filtered hot and the cooled filtrate extracted with ether in a liquid-liquid extractor for four days. The residue from the ether was treated with dilute sodium carbonate solution. The insoluble portion was sublimed at 170°/2 mm. to yield a material which was recrystallized from ethanol to give needles, m. p. 263–264°. A mixed m. p. with a sample of N-methyl-*m*-hemipinimide prepared from authentic *m*-hemipinic acid[1] gave no depression.

Potassium Permanganate Oxidation of Norargemonine.—A 1.02-Gm. sample of norargemonine was dissolved in 50 ml. of 5% hydrochloric acid. To this was added 50 ml. of a 10% sodium hydroxide solution and the mixture allowed to cool. A 5% potassium permanganate solution was added dropwise until the color persisted for one hour. The excess permanganate color was discharged by the addition of ethanol. The reaction mixture was filtered and the insoluble residue washed with hot water. The combined filtrates were acidified with dilute hydrochloric acid and extracted with ether for four days in a liquid-liquid extractor. The residue from the ether was completely soluble in 5% sodium carbonate solution with effervescence. The sodium carbonate solution was filtered, acidified, and extracted with ether. The residue from the ether weighed 0.4 Gm.

N-Ethylimide of Acid.—Two hundred milligrams of the acidic residue was dissolved in 4 ml. of 33% aqueous ethylamine and the solution concentrated to dryness. This residue was heated at 180° for ten minutes then sublimed at 180°/5 mm. The white sublimate was recrystallized from methanol to yield 34 mg. of white needles, m. p. 223–225° (capillary). A mixed m. p. with N-ethyl-*m*-hemipinimide prepared from authentic *m*-hemipinic acid gave no depression.

N-Methylimide.—The remaining 200 mg. of acidic residue was dissolved in 4 ml. of a 40% aqueous methylamine solution and treated as before to yield 60 mg. of a white crystalline material, m. p. 262–263° (capillary). A mixed m. p. with N-methyl-*m*-hemipinimide prepared from authentic *m*-hemipinic acid gave no depression.

Ethylation of Bisnorargemonine.—A 1.25-Gm. sample of bisnorargemonine was dissolved in 200 ml. of dichloromethane with the aid of 20 ml. of dry methanol. The solution was cooled to 5° and a freshly prepared solution of 4 Gm. diazoethane in ether was added. The flask was allowed to come to room temperature over a period of several hours. The solvents were removed and the residue taken up in 5% hydrochloric acid. The acid solution gave a positive Mayer's test. It was washed with ether, made strongly alkaline, and extracted with chloroform. The residue from the chloroform weighed 1.38 Gm. Attempts to purify the product were unsuccessful.

Manganese Dioxide Oxidation of Ethylated Bisnorargemonine.—The impure ethylated bisnorargemonine, 1.3 Gm., was dissolved in dilute acid. Manganese dioxide, 1.5 Gm., was added and the suspension heated on a steam bath with stirring for five hours. The suspension was filtered hot, cooled, and then extracted with ether in a liquid-liquid extractor for fifteen hours. The residue from the ether weighed 200 mg.

Acidic Fraction.—The residue was treated with 5% sodium carbonate to dissolve the acidic constituents. The insoluble material was worked up as the neutral fraction. Half of the sodium carbonate solution was acidified, extracted with ether, and treated with a methylamine solution as before. The N-methylimide was sublimed and recrystallized to give needles, m. p. 193–194°. The remaining half of the sodium carbonate solution was treated similarly and the acid converted to the N-ethylimide, m. p. 203.5–205°. The reported (13) m. p. of N-ethyl-4-methoxy-5-ethoxy phthalimide is 205°.

Anal.—Calcd. for $C_{13}H_{15}NO_4$: C, 62.63, H, 6.07; N, 5.62. Found: C, 63.18; H, 6.47; N, 5.81.

Neutral Fraction.—The sodium carbonate-insoluble portion of the oxidation mixture was sublimed at 180°/5 mm. The yellow-white product was recrystallized from ethanol to yield a product, m. p. 193–194°. A mixed m. p. with the previously prepared N-methylimide of the acid showed no depression.

Permanganate Oxidation of Ethylated Bisnorargemonine.—Three hundred milligrams of impure ethylated bisnorargemonine was dissolved in dilute hydrochloric acid, and then the solution made basic with 20% sodium hydroxide solution. A 5% potassium permanganate solution was added dropwise until the color persisted. The color was discharged in the usual manner. The solution was filtered, the filtrate acidified, and extracted with ether. The residue from the ether weighed 125 mg. This was treated with an ethylamine solution and heated and sublimed as before. The product, m. p. 203.5–205°, gave no depression with the N-ethyl-4-methoxy-5-ethoxy phthalimide obtained in the manganese dioxide oxidation of ethylated bisnorargemonine.

Argemonine Methiodide.—One gram of argemonine methiodide in 30 ml. of water was treated with an excess of fresh silver oxide and stirred until all of the silver iodide had precipitated. The solution was filtered and sodium hydroxide added to make a 20% solution. This was heated to boiling for ninety minutes and then cooled. An oil formed on the sur-

[1] Kindly supplied by R. H. F. Manske, Dominion Rubber Co., Guelph, Ontario, Canada.

face and hardened as the solution cooled. This oil was extracted with ether, dried, and concentrated to a tan, translucent oil weighing 430 mg. Attempts to purify the residue were unsuccessful, $[\alpha]_D^{24}$ + 53.6°. Infrared analysis showed no terminal vinyl group absorption at 910 and 990 cm.$^{-1}$.

Methiodide.—Fifty milligrams of the oily methine was converted to the methiodide, m. p. 138–140°.

Hydrochloride.—Two hundred milligrams was converted to the hydrochloride by treating a dry ether solution of the methine with dry ether saturated with hydrogen chloride. The product was recrystallized from absolute ethanol to give 200 mg. of crystals, m. p. 206–207°. A solution of the hydrochloride salt decolorized a bromine solution with no liberation of hydrogen bromide. Infrared analysis showed weak terminal vinyl group absorption at 910 and 990 cm.$^{-1}$.

Picrate.—One hundred milligrams of the methine base was converted to the picrate, m. p. 189–192°.

Anal.—Calcd. for $C_{28}H_{30}N_4O_{11}$: C, 56.20; H, 5.05. Found: C, 56.08; H, 5.08.

Nitrogen-Free Compound.—Three hundred milligrams of argemonine methine methiodide in 18 ml. of water was treated with an excess of fresh silver oxide and stirred until all of the silver iodide had precipitated. The solution was then filtered and the filtrate warmed for one hour. During this time the odor of trimethylamine was detected. A quantity of this gas was passed into alcoholic picric acid and the resulting picrate purified, m. p. 203–204°. A mixed melting point with authentic trimethylamine picrate gave no depression.

The precipitate which formed in the reaction mixture was removed by filtration and recrystallized from methanol, m. p. 165–167° (capillary), $[\alpha]_D^{27}$ + 15.7°. A sodium fusion test for nitrogen was negative.

Anal.—Calcd. for $C_{20}H_{22}O_5$: C, 70.16; H, 6.48; OCH_3, 36.26. Found: C, 70.27; H, 6.57; OCH_3, 35.60.

A sample was dried *in vacuo* and found to have lost no weight. Infrared analysis showed no hydroxyl group absorption at 3,600–3,300 cm.$^{-1}$ or carbonyl group absorption at 1,800–1,600 cm.$^{-1}$. Absorption at 1,095 and 1,055 cm.$^{-1}$ might be attributed to the presence of a pyran ring.

Stability Observations.—A year-old sample of the nitrogen-free compound was found to melt at 155–163°. A fresh sample which had been crystallized from aqueous methanol melted at 143–157°. A 100-mg. sample of this material was refluxed with water for five hours, m. p. 135–143°. Subsequent recrystallizations succeeded only in giving lower melting points with a broader range, indicating increasing decomposition of the original material.

Acid Hydrolysis of the Nitrogen-Free Compound.—One hundred mg. of the fresh nitrogen-free compound was dissolved in 10 ml. of ethanol with heat. After cooling, 5 ml. of concentrated hydrochloric acid was added. The solution became purple and then pale yellow in color. The reaction mixture was refluxed gently for twenty minutes. Crystals soon formed which were filtered off when the solution cooled. The product was recrystallized from ethanol, m. p. 219–220°. A sodium fusion and subsequent test for chlorine was positive.

Anal.—Calcd. for $C_{20}H_{21}ClO_5$: C, 66.57; H, 5.87; Cl, 9.83; OCH_3, 34.40; C—CH_3, none. Found: C, 66.64; H, 5.77; Cl, 9.55; OCH_3, 34.58; C—CH_3, none. Infrared analysis showed the absence of a vinyl group.

Hydrogenation of Argemonine Methine.—Argemonine methine, 1.5 Gm., was dissolved in 25 ml. of ethanol and 5 ml. of concentrated hydrochloric acid. This solution was catalytically hydrogenated with platinum oxide at 40 pounds pressure. The hydromethine hydrochloride from the reaction mixture was sublimed *in vacuo* and recrystallized from ethanol, m. p. 224–227°, $[\alpha]_D^{23}$ + 75.7°.

Anal.—Calcd. for $C_{20}H_{16}ClNO_4$: C, 64.78; H, 7.41. Found: C, 64.35; H, 7.60.

Hydromethine Base.—The crystalline hydrochloride was converted to the free base with sodium carbonate. It was a yellow oily material which could not be crystallized.

Methiodide of Hydremethine.—One gram of the methine base was converted to the methiodide, m. p. 182–184°.

Anal.—Calcd. for $C_{21}H_{22}INO_4$: C, 53.80; H, 6.28. Found: C, 53.66; H, 6.16.

Elimination of Nitrogen.—A 1.45-Gm. sample of the hydromethine methiodide was dissolved in 30.0 ml. of water and an excess of fresh silver oxide added. The mixture was stirred to precipitate all of the silver iodide, filtered, and the filtrate made up to a 20% solution of sodium hydroxide. This solution was heated for five hours. A yellow oil formed on the surface which hardened upon cooling. The oil was sublimed at 160–190°/2 mm., recrystallized from ethanol to give shiny plates, m. p. 156–158°. A sodium fusion and subsequent test for nitrogen was negative. During the heating phase, a gas was evolved which was identified as trimethylamine from a comparison of the picrates.

Anal.—Calcd. for $C_{20}H_{22}O_4$: C, 73.62; H, 6.80; OCH_3, 38.02. Found: C, 73.98; H, 7.14; OCH_3, 37.30.

Oxidation of Nitrogen-Free Compound.—A suspension of 360 mg. of the N-free ether in 25 ml. of water was warmed and 25 ml. of 5% potassium permanganate added. The reaction mixture was heated for one hour, cooled, and the excess permanganate reduced with methanol. The suspension was cooled, acidified with 10 ml. of 50% sulfuric acid, and the color discharged with powdered sodium bisulfite. A fine white precipitate remained which was removed by centrifugation and crystallized from methanol, m. p. 254–254.5° with effervescence. The effervescence was found to be due to evolution of a mole of water by passing the gas evolved at the melting point from a weighed sample through a carbon-hydrogen train. The acid gave a positive fluorescein test.

Anal.—Calcd. for $C_{12}H_3(OCH_3)_4(COOH)_3$: C, 56.16; H, 4.46; OCH_3, 30.55; active H, 0.74; Grignard consumption without gas evolution, 1.48. Found: C, 56.56; H, 4.43; OCH_3, 30.13; active H, 0.77; Grignard consumption without gas evolution, 1.52.

Methyl Ester.—One hundred milligrams of the acid in 100 ml. of absolute methanol was treated with dry hydrogen chloride gas bubbled in at room temperature. After five hours the solution was stoppered and allowed to stand for two days. Two crops of crystals were collected and recrystallized from ethanol, m. p. 315–315.5°.

Anal.—Calcd. for $C_{28}H_{24}O_{10}$: C, 58.92; H, 5.39. Found: C, 58.40; H, 5.63.

Decarboxylation.—One hundred milligrams of the acid was heated slowly to 250° with 0.4 Gm. of copper-bronze and 5 ml. of freshly distilled quinoline. Dry nitrogen was passed through the apparatus sweeping all evolved gases through a conventional carbon-hydrogen type train. The weight of carbon dioxide was found to be 10 mg. as compared to the calculated weight for carbon dioxide from one carboxyl group of 10.8 mg. Attempts to purify the main decarboxylation product were unsuccessful.

O-Methyl Corydine Methine.—One gram of corydine hydrochloride (Eastman Kodak Co.) was converted to the free base, then completely methylated with dimethyl sulfate according to Gadamer (14). The resulting impure O-methyl corydine methosulfate was digested with 30% sodium hydroxide solution for four hours to produce an oil as reported by Gadamer.

Picrate.—The picrate was prepared according to the conventional procedure, m. p. 182.5–184°. A mixed melting point with argemonine methine picrate showed a marked depression.

Methiodide.—The methiodide was prepared according to Gadamer's procedure (14). It sintered above 260°. A mixed melting point with argemonine methine methiodide gave a marked depression.

Corydine N-Free Compound.—The corydine methine methiodide was converted to the nitrogen-free compound according to Gadamer's procedure using a strong sodium hydroxide solution. A golden-yellow oil was obtained as reported. It could not be crystallized.

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2-Deoxystreptamine Derivatives

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A group of N- and O-substituted 2-deoxystreptamine derivatives and their salts, represented by types I and II, have been synthesized for biological evaluation. Methods of their syntheses are discussed.

KANAMYCIN, discovered and characterized by Umezawa and co-workers (1, 2), is a water-soluble basic antibiotic which is active against many mycobacteria and Gram-positive and Gram-negative organisms. By boiling it for forty-five minutes in 6 *N* hydrochloric acid, kanamycin was hydrolyzed to 1,3-diamino-4,5,6-trihydroxycyclohexane (2-deoxystreptamine) dihydrochloride by Hooper and co-workers (3) while engaged in degradative studies which have led to an essentially complete structural elucidation for this important antibiotic (4).

It is noteworthy that several other antibiotics are known to contain 2-deoxystreptamine as a

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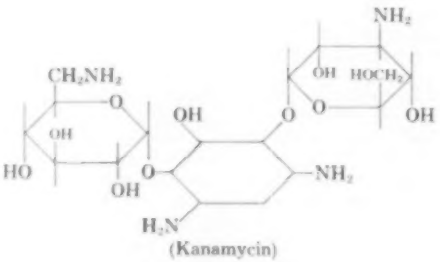
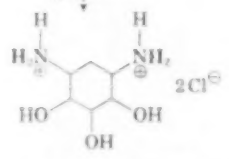


(Kanamycin)

8N HCl, 45 min.



1,3-Diamino-4,5,6-trihydroxycyclohexane dihydrochloride
(2-Deoxystreptamine) dihydrochloride

structural component, including the neomycins (5, 6), hydroxymycin (7), kanamycin *B* (8), and paromomycin (9).

In the course of our search for new compounds having improved antimicrobial activity, fifteen N- and O-substituted 2-deoxystreptamine derivatives were synthesized for biological evaluation. These include acetyl and benzoyl derivatives, alkylated compounds resulting from the Leuckart reaction, and several Schiff bases. The latter were reduced catalytically and the products used in further alkylation or acylation reactions.

The pentaacetyl product, which had been prepared previously by Hooper and co-workers (3), was hydrolyzed with concentrated ammonia solution to give 1,3-bisacetyl-2-deoxystreptamine (I) (Table I). Benzoylation of 2-deoxystreptamine base with benzoyl chloride gave the corresponding dibenzoyl derivative (II). The Schiff base IV, prepared quantitatively from benzaldehyde and 2-deoxystreptamine, was reduced with hydrogen in the presence of Raney nickel to produce III. The Schiff base V from *o*-methoxybenzaldehyde and 2-deoxystreptamine was made by following the same procedure employed for the preparation of IV. 1,3-Dibenzylidene-4,5,6-triacetyl-2-deoxystreptamine (VI) was prepared by acylation of IV in dry pyridine with acetic anhydride, refluxing the mixture for four hours. 1,3 - Diacetyl - 4,5,6 - phenylcarbamyl - 2 - deoxystreptamine (VII) was made from compound I by boiling the latter with phenyl isocyanate for seven minutes. When 2-deoxystreptamine was refluxed with *p*-nitrobenzoyl chloride (1:2 molar ratio) in pyridine for ten minutes, a tetra -*p*-nitrobenzoyl product was isolated. After instrumental studies, a provisional structure (1,3,4,6-tetra-*p*-nitrobenzoyl-2-deoxystreptamine) was assigned. The tetra-*p*-nitrobenzoyl product was then reduced to the tetra-*p*-aminobenzoyl derivative (VIII). 1,3-Dibenzyl-1,3-dimethyl-2-deoxystreptamine (IX) was prepared by refluxing III with formaldehyde in the presence of formic acid (Leuckart reaction). When compound III was caused to react with an excess of phenyl isocyanate, 1,3 - dibenzyl - 1,3,4,5,6 - pentaphenylcarbamyl-2-deoxystreptamine (X) resulted.

Compound XI (Table II) was prepared by the condensation of 2-deoxystreptamine and isobutyraldehyde in ethanol, followed by reduction of the product with hydrogen over Raney nickel and acidification to obtain the hydrochloride. When 2-deoxystreptamine was refluxed with formic acid and formaldehyde (1:4 molar ratio) compound XIII was isolated as the hydrochloride salt, which was then acetylated to give compound XII. Compound V was treated with ethanolic hydrogen chloride to obtain the corresponding salt. 1,3 - Guanyl - 2 - deoxystreptamine sulfate (XV) was made from the 2-deoxystreptamine and 2-methylisothiourea sulfate in aqueous solution.

EXPERIMENTAL

Synthesis.[1]—*1,3 - Bis - acetyl - 2 - deoxystreptamine* (I).—Pentaacetyl-2-deoxystreptamine, 11.2 Gm. (0.035 mole); 29.9% ammonia, 11.0 Gm.; and 150 ml. distilled water were refluxed for one hour. The solution was evaporated nearly to dryness, diluted with 10 ml. methanol, and stirred until a crystalline deposit formed. The yield of white solid, m. p. 292–293°, was 4.5 Gm. (61%).

After recrystallization from dimethyl formamide, the product melted at 283–285°.

1,3 - Dibenzoyl - 2 - deoxystreptamine (II).—Eight grams (0.05 mole) of 2-deoxystreptamine was refluxed for ten minutes in 25 ml. of benzoyl chloride. The mixture was cooled to room temperature and 25 ml. of petroleum ether was added. The crystals were filtered off and washed twice with 15-ml. portions of petroleum ether, then successively with 20% sodium carbonate solution, water, and finally, with ether. The product was dried and a yield of 7.0 Gm. (55%), m. p. 292–300°, was observed. The high carbon analysis indicates the probable presence of impurity.

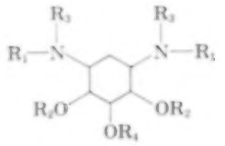
1,3-Dibenzylidene-2-deoxystreptamine (IV) *and 1,3-Dibenzyl-2-deoxystreptamine* (III).—2-Deoxystreptamine, 48.6 Gm. (0.3 mole) and 63.6 Gm. (0.6 mole) of benzaldehyde in 150 ml. of methanol were refluxed for ten minutes and then allowed to stand at room temperature for two hours. The mixture was evaporated to dryness under reduced pressure. The residue was dissolved in benzene and precipitated with petroleum ether. The yield of desired product was 85.0 Gm. (84.2%), m. p. 94–96°. The crystals were then reduced in alcohol, using Raney nickel as the catalyst. The yield was 84.5 Gm. (98.0%), m. p. 145–147°.

1,3 - Di - o - methoxybenzylidene - 3 - deoxystreptamine (V).—*o*-Methoxybenzaldehyde, 100.0 Gm. (0.74 mole) and 40 Gm. (0.25 mole) of 2-deoxystreptamine in 50 ml. of methanol were refluxed for forty-five minutes, cooled, and poured into 2 L. of ice water. Crystals formed on standing. The water was decanted. The residual crystals, after drying, were dissolved in benzene; the benzene solution was dried over Drierite, and the product was precipitated out by the addition of petroleum ether. A yield of 83.2 Gm. (84%), m. p. 186–188°, was obtained.

1,3 - Dibenzylidene - 4,5,6 - triacetyl - 2 - deoxystreptamine (VI).—1,3-Dibenzylidene-2-deoxystreptamine, 13.6 Gm. (0.04 mole), in 200 ml. of dry pyridine and 20 ml. of acetic anhydride was refluxed for four hours, during which time an orange solution formed. The mixture was cooled and 180 ml. of petroleum ether added. The resulting crystals were removed by filtration and washed twice with 30 ml. of petroleum ether. The product was

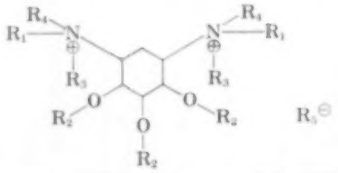
[1] Properties and analyses of the new compounds are shown in Tables I and II. All melting points are uncorrected. Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, New York, N. Y.

TABLE I.—2-DEOXYSTREPTAMINES, TYPE I



No.	R_1	R_2	R_3	R_4	M. P., °C.	Yield, %	Formula	Mol. Weight	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
I	CH_3CO	H	H	H	292–293	61	$C_{15}H_{18}N_2O_5$	246.26	48.77	48.90	7.36	7.46	11.37	11.72
II	C_6H_5CO	H	H	H	292–300	55	$C_{20}H_{22}N_2O_5$	370.40	64.85	66.23	5.98	6.12	7.56	7.71
III	$C_6H_5CH_2$	H	H	H	145–147	98	$C_{20}H_{26}N_2O_3$	342.43	70.15	69.91	7.65	7.85	8.18	8.19
IV	C_6H_5CH	H	...	H	79–80	84	$C_{20}H_{22}N_2O_3$	338.40	70.98	70.85	6.55	6.89	8.28	8.00
V	o-$H_2COC_6H_4CH$	H	...	H	186–188	84	$C_{22}H_{26}N_2O_5$	398.45	66.31	66.41	6.57	6.50	7.02	7.20
VI	C_6H_5CH	CH_3CO	...	CH_3CO	170–171	66	$C_{28}H_{28}N_2O_6$	464.52	67.22	67.66	6.07	6.19	6.03	6.26
VII	CH_3CO	C_6H_5NHCO	H	C_6H_5NHCO	293–295	90	$C_{31}H_{33}N_5O_5$	603.64	61.68	62.38	5.50	5.71	11.60	11.58
VIII	p-$H_2NC_6H_4CO$	p-$H_2NC_6H_4CO$	H	H	295–297	75	$C_{34}H_{34}N_6O_7$	638.68	63.93	64.63	5.36	5.38	13.15	13.35
IX	$C_6H_5CH_2$	H	CH_3	H	51–52	65	$C_{22}H_{30}N_2O_3$	370.49	71.32	70.23	8.16	8.02	7.56	7.30
X	$C_6H_5CH_2$	C_6H_5NHCO	C_6H_5NHCO	H	233–237	97	$C_{55}H_{51}N_7O_5$	938.06	70.42	70.32	5.48	5.43	10.45	10.55

TABLE II.—2-DEOXYSTREPTAMINES, TYPE II



No.	R_1	R_2	R_3	R_4	R_5	M. P., °C.	Yield, %	Formula	Mol. Weight	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found	Chlorine Calcd.	Chlorine Found
XI	$(CH_3)_2CHCH_2$	H	H	H	Cl	294–296	66	$C_{14}H_{27}Cl_2N_3O_5$	347.32	48.41	48.06	9.28	9.63	8.06	7.84	20.41	19.99
XII	CH_3	CH_3CO	CH_3	H	Cl	263–265	87	$C_{16}H_{27}Cl_2N_3O_7$[a]	435.35	44.14	44.85	7.40	7.43	6.43	6.47	16.29	16.75
XIII	CH_3	H	CH_3	H	Cl	263–265	79	$C_{10}H_{25}Cl_2N_3O_5$	327.25	36.70	36.57	8.62	8.58	8.56	8.70	21.66	21.77
XIV	o-$H_2COC_6H_4CH_2$	H	H	H	Cl	213–215	94	$C_{22}H_{37}Cl_2N_3O_5$	475.20	55.54	55.31	6.79	7.07	5.90	6.06	14.92	14.35
																Sulfur	
XV	H_2NCNH	H	H	H	$SO_4^{\ominus}$	165–170	13	$C_5H_{20}N_6O_7S$	344.34	27.90	28.17	5.85	5.99	24.40	24.10	9.31	9.28

[a] One mole of water present.

recrystallized from a benzene-petroleum ether mixture, yield 12.2 Gm. (66%), m. p. 168–170°.

1,3 - Diacetyl - 4,5,6 - tri - phenylcarbamyl - 2-deoxystreptamine (VII).—1,3-Diacetyl-2-deoxystreptamine (I), 3.5 Gm. (0.014 mole) was dissolved in 400 ml. of dry pyridine. Phenyl isocyanate was added and the mixture was refluxed for seven minutes. The reaction mixture was cooled; the resulting crystals were removed by filtration and washed with 40 ml. of petroleum ether. Yield of dried product was 7.5 Gm. (90%), m. p. 293–295°.

1,3,4,6 - p - Aminobenzoyl - 2 - deoxystreptamine (VIII).—2-Deoxystreptamine, 16.2 Gm. (0.10 mole), 37.0 Gm. (0.20 mole) of *p*-nitrobenzoyl chloride, and 60 ml. of pyridine were refluxed for ten minutes. The solution was poured into 400 ml. water, filtered, and the impurities removed from the crude product by extracting with 300 ml. of hot ethylene glycol monomethylether. The yield of white crystals was 11.3 Gm. (30.0%), m. p. >310°. The tetra-*p*-nitrobenzoyl-2-deoxystreptamine thus obtained was reduced with hydrogen over Raney nickel at an initial pressure of 49 pounds. The product was recrystallized from methanol. The yield was 8.5 Gm. (75%), m. p. 295–297°.

1,3 - Dibenzyl - 1,3 - dimethyl - 2 - deoxystreptamine (IX).—1,3-Dibenzyl-2-deoxystreptamine (II), 11.4 Gm. (0.033 mole); 7.5 Gm. (0.088 mole) 35% formaldehyde; and 9.0 Gm. (0.17 mole) of 88% formic acid were refluxed for sixteen hours. Six milliliters of concentrated hydrochloric acid were added and the solution evaporated until a thick syrup was obtained. The product was dissolved in 150 ml. of water and made alkaline with 10% sodium hydroxide solution; the precipitate thus obtained was dissolved in ether, dried over anhydrous potassium carbonate, and the ether removed by distillation. A colorless oil was obtained which became a flaky solid on pumping at high vacuum for several hours. Yield was 8.0 Gm. (65%), m. p. 51–52°.

1,3 - Dibenzyl - 1,3,4,5,6 - pentaphenylcarbamyl-2-deoxystreptamine (X).—1,3-dibenzyl-2-deoxystreptamine, 8.5 Gm. (0.03 mole) was mixed with 18.0 Gm. (0.15 mole) of phenyl isocyanate. The temperature of the reaction mixture rose to 40°; external heat was applied and the mixture was refluxed for ten minutes. The phenyl isocyanate was removed under reduced pressure. The remaining crystals were washed twice with 40-ml. portions of petroleum ether. Yield, 27.3 Gm. (97%), m. p. 233–237°.

1,3-Diisobutyl-2-deoxystreptamine Dihydrochloride (XI).—2-Deoxystreptamine, 16.2 Gm. (0.10 mole); 15.0 Gm. (0.21 mole) of isobutyraldehyde; and 50 ml. of absolute ethyl alcohol were refluxed for one hour. The solid was removed under reduced pressure and half of the crude product was reduced with hydrogen over Raney nickel at an initial pressure of 49 pounds. After filtration of the catalyst, the solution was evaporated to 30 ml., acidified with anhydrous hydrogen chloride in absolute ethanol, and this solution was finally diluted with 500 ml. of absolute ether to yield a crystalline product which was filtered, washed with ether, and dried in an oven. Yield of white solid was 11.4 Gm. (66%), m. p. 294–296°, with decomposition.

1,1,3,3 - Tetramethyl - 4,5,6 - triacetyl - 2 - deoxystreptamine Dihydrochloride (XII).—1,1,3,3-Tetramethyl-2-deoxystreptamine dihydrochloride (XIII), 11.6 Gm. (0.04 mole); 50 ml. of acetic anhydride; and 50 ml. of dry pyridine were refluxed for five hours. The mixture was chilled overnight, filtered, and the solid thus obtained was washed with acetone. The yield of light gray crystals was 14.5 Gm. (87%), m. p. 265–266°.

1,1,3,3 - Tetramethyl - 2 - deoxystreptamine Dihydrochloride (XIII).—2-Deoxystreptamine, 16.2 Gm. (0.10 mole); 51.0 Gm. (1.0 mole) of 88% formic acid; and 37.6 Gm. (0.44 mole) of 35% formaldehyde were refluxed for three hours. The mixture was acidified with 20 ml. concentrated hydrochloric acid and evaporated on the steam bath. The product was triturated with acetone, and finally boiled with 300 ml. absolute ethanol until crystallization occurred. The yield of nearly colorless crystals was 22.8 Gm. (78%), m. p. 263–265°, with previous softening at 105–110°, followed by resolidification (indicative of loss of water). Recrystallization of this material from 95% ethanol produced no change in melting point.

1,3 - Di - o - methoxybenzyl - 2 - deoxystreptamine Dihydrochloride (XIV).—To a 40.0-Gm. (0.1 mole) portion of 1,3-di-*o*-methoxybenzyl-2-deoxystreptamine in 100 ml. of ethanol solution was added 200 ml. of 50% ethanolic hydrochloride until a clear acidic mixture was obtained. To this mixture 1,000 ml. of ether was added. The reaction mixture was cooled, and the crystals which formed were removed by filtration; yield 44.6 Gm. (94%), m. p. 213–215°, of product.

1,3-Di-guanyl-2-deoxystreptamine Sulfate (XV)—To 11.34 Gm. (0.07 mole) of 2-deoxystreptamine was added, portionwise, 19.46 Gm. (0.07 mole) of 2-methylisothiourea sulfate in aqueous solution, 50 ml., over a period of twenty-four hours, maintaining the temperature at 70–75°. At this point, 1.1 Gm. more of 2-methylisothiourea was added, and the mixture was heated another twenty-four hours at the same temperature. It was then cooled to 0°, 50 ml. of acetone added, and filtered. The crystals which formed were washed three times with 1 *N* ammonia solution, 10 ml. acetone, and dried in high vacuum at 100°. Yield, 4.7 Gm. (13%), m. p. 165–170°.

Biological Activity.—None of the compounds described has been found to possess appreciable antimicrobial activity or noteworthy pharmacological properties in tests conducted to date.

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Application of Paper Chromatography and Electrophoresis to the Assay of Pharmaceutical Products

By HERBERT SCHRIFTMAN and ROBERT C. SHULTZ

Results comparable to conventional colorimetric and spectrophotometric methods have been obtained by paper chromatographic and electrophoretic methods. In several instances, where stability studies are involved or when the combination of drugs do not lend themselves to assay by the usual methods, it is concluded that the chromatographic and/or electrophoretic procedures are a practical applicable solution. Some of the modifications that are needed for applying these chromatographic and electrophoretic methods to pharmaceutical assays are discussed. In general, separations of the various compounds are accomplished by ascending or descending paper partition chromatography, by selection of appropriate solvent systems, or by paper electrophoresis with selected pH buffer systems. The developed papergrams are sprayed with an appropriate color reagent and assayed quantitatively by photoelectric densitometry. The compounds treated in this manner are: phenylephrine hydrochloride, chlorpheniramine maleate, dextromethorphan hydrobromide, N-acetyl-*p*-aminophenol, meperidine hydrochloride, atropine, ethinyl estradiol, procaine and tetracaine hydrochlorides.

DRUGS THAT HAVE been in general use a long time are now becoming available in preparations containing other active compounds. In handling the assay of these combinations of drugs, the usual methods, such as the ultraviolet spectrophotometric and colorimetric assays, become laborious and unwieldy because additional filtering and extraction steps are needed for their successful application. In addition, many of the assays fail to detect chemical modifications of the drugs that may occur when the various experimental formulations of the combination products are subjected to accelerated stability studies. This was illustrated in the case of phenylephrine in a previous publication (1).

In the paper chromatographic and electrophoretic methods presented here, results comparable to established colorimetric and spectrophotometric procedures have been obtained whenever the conventional methods have been applicable to freshly prepared products. In instances where stability studies are involved or when the combination drugs do not lend themselves to assay by the more conventional procedures, it is shown that the chromatographic and/or electrophoretic methods are desirable routine analytical procedures.

In general, separations of the various compounds are accomplished through ascending and descending paper partition chromatography by selection of appropriate solvent systems or by paper electrophoresis with selected pH buffer systems. The papergrams are sprayed with an appropriate color developing reagent and assayed quantitatively by photoelectric densitometry.

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EXPERIMENTAL

Combination of Phenylephrine Hydrochloride Chlorpheniramine Maleate, and Dextromethorphan Hydrobromide.—A two-dimensional paper chromatographic method was applied in the assay of a pharmaceutical preparation containing phenylephrine hydrochloride, chlorpheniramine maleate, and dextromethorphan hydrobromide, as illustrated by the diagram in Fig. 1. An ascending chromatographic technique was used. Standard spots equivalent to 20 mcg. of chlorpheniramine, 75 mcg. of phenylephrine, and 100 mcg. of dextromethorphan were applied to the paper while the sample solution was spotted so as to approximate these values according to claimed amounts. Extraction and preparation of sample solution was similar to that described below in the paper electrophoretic method.

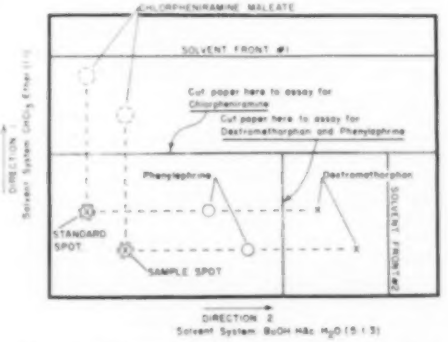

Fig. 1.—Diagram of two-dimensional paper chromatographic method, for assay of chlorpheniramine, dextromethorphan, and phenylephrine.

Both the sample and standard spots were placed in such a way on the paper that the migration of each compound did not overlap or interfere with any other in the course of their ascent. The Whatman No. 1 paper, after the spots were applied with a micropipet, was rolled up into a cylinder, stapled or stitched together, and placed in a solvent dish containing solvent system No. 1 [chloroform: ether (1:1)], which separated the chlorpheniramine maleate from the other two active ingredients in about two hours.

The paper was then unrolled and cut in such a way as to remove the standard and sample spots of the chlorpheniramine maleate for quantitative assay. This component was found to have moved up very close to the solvent front. The paper was turned at right angles and again rolled up into a cylinder and placed in a dish containing solvent system No. 2 [butanol:acetic acid:water (5:1:3)] in another pipet jar. The migration time required to separate phenylephrine and dextromethorphan was about three or four hours. The two drugs were located by observing the fluorescent properties of phenylephrine, employing a long wave U. V. scanning lamp. This paper was then cut as required to permit separate quantitative assay of the active ingredients. The dextromethorphan and chlorpheniramine were located and assayed based on the characteristic orange-red spots developed by them when sprayed with modified Dragendorff's reagent (iodobismuthate) as described before (1, 2). All the spots were assayed for relative total color intensity with a Photovolt densitometer, model 525, equipped with a semiautomatic plotting attachment and narrow band color filters. The total color density readings of the reference standard and sample spots were compared by direct proportion. A 525 mμ color filter in the densitometer was selected for the assay of the chlorpheniramine maleate and dextromethorphan hydrobromide spots while a 440 mμ filter was used for the phenylephrine assay.

In order to run more assays, the only limiting factor is the diameters of the jars or solvent dishes since two or more cylinders can be nested at the same time by fitting several cylinders, each of smaller diameter, inside a larger one. The surfaces of the papers must not touch each other. Table I shows how well assay results by this method compare with spectrophotometric and colorimetric methods. The conventional method requires a colorimetric analysis of the phenylephrine (3) followed by bicomponent spectral analysis for the other two ingredients and must include corrections for interference from base materials.

A paper electrophoretic method was also applied and compared to the generally applied method. This was conducted as follows: an accurately weighed powdered sample, equivalent to the theoretical contents of five tablets containing 2 mg. of chlorpheniramine maleate, 7.5 mg. of phenylephrine hydrochloride, and 10 mg. of dextromethorphan per tablet was transferred to a 25-ml. volumetric flask and diluted to the mark with absolute alcohol. The mixture was well shaken and undissolved material was allowed to settle to the bottom before spotting sample. A standard solution containing known amounts of the above active ingredients was likewise prepared in absolute alcohol. One-inch strips (62 cm. in length) of Whatman No. 3 MM paper were prepared by immersing them in the electrolyte solution of pH 4 acetate buffer of ionic strength 0.1 and were blotted between a folded sheet of filter paper. The strips were placed in an electrophoresis apparatus (NEC-455-18-inch electrophoresis apparatus with power supply unit EC 403).[1]

TABLE I.—COMPARATIVE ASSAY RESULTS

Samples	Chromatographic Method, % of Claim	Conventional Colorimetric and Spectrophotometric Method, %
Chlorpheniramine maleate	103.4	105.1
Dextromethorphan hydrobromide	99.6	100.5
Phenylephrine HCl	100.0	102.4
	Electrophoretic Method	
Chlorpheniramine maleate	101.2	103.0
Dextromethorphan hydrobromide	99.6	98.1
Phenylephrine HCl	104.8	106.2

Using four separate strips of prepared papers, each of two were spotted with 0.025 ml. of each of the standard solution and the sample solution prepared from the powdered mixtures, by adding the spots in 0.005-ml. increments with a micropipet. The spots were placed closer to one end of the paper (8 to 10 cm. from the end) for longer migration. The end used was that which corresponded with the "red-marked" side of the terminal insert plug from the power supply which could be changed daily from one side to another of the terminal. The apparatus was then set up according to the operating directions, a voltage of 600 gradually applied, and migration allowed to proceed at this voltage for five hours, using ice water circulating through the plates. It was also run at 150 volts overnight (sixteen–eighteen hours) in a refrigerator room (5°). At the end of the appropriate time, the strips were removed and air dried. One set of strips (standard and sample) was sprayed with *p*-sulfanilic acid reagent (1) to detect the phenylephrine spots which are an intense yellow. The other strips were sprayed with Dragendorff's reagent (1, 2) to detect the orange-red spots of dextromethorphan and chlorpheniramine. The total color density of the spots were analyzed and compared as described previously for the paper chromatographic procedure. Here again (see Table I) comparison with the established method was favorable. The paper electrophoretic method, however, became the more suitable means of assay, after N-acetyl-*p*-aminophenol was added to the combination of drugs just described.

The assay was conducted as described above but an additional spot of N-acetyl-*p*-aminophenol showed on the strips sprayed with the *p*-sulfanilic acid as a pink-violet spot and had a slower migration than the intense yellow spot of phenylephrine. The N-acetyl-*p*-aminophenol spot was assayed as above but a 485 mμ color filter was used. The amounts of the other drugs were the same per capsule as in the tablet preparations mentioned above, but the

[1] E-C Apparatus Co., Swarthmore, Pa.

N-acetyl-*p*-aminophenol was present in the amount of 194.4 mg. per capsule. The two lower electrophoretic strips in Fig. 2 show the relative migration of the four spots representing the drugs in this complex mixture.

Meperidine Hydrochloride, Alone and in Combination with Other Compounds.—A paper electrophoretic method was employed for the assay of meperidine hydrochloride, alone and in combination with other drugs in such types of preparations as tablets and injection solutions. The method was used as follows: powdered tablets approximately equivalent to 250 mg. of meperidine hydrochloride were transferred to a 25-ml. volumetric flask. Absolute alcohol or distilled water was added to the powder (depending on whether or not certain ingredients were used in the tablet formulation) and diluted to mark. This mixture was shaken thoroughly for at least fifteen minutes, mechanically or by hand. The solution was allowed to settle or it was filtered through a dry paper, and with the aid of a micropipet, two equal quantities of 0.005 ml. of these solutions were applied to Whatman No. 3 MM paper.

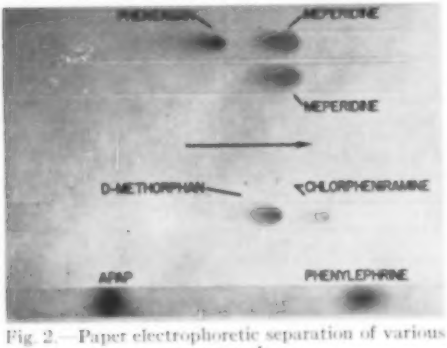

Fig. 2.—Paper electrophoretic separation of various compounds.

For preparation of paper and subsequent electrophoretic treatment of these strips and the assay of the spots, see previous section describing the use of paper electrophoresis for chlorpheniramine and dextromethorphan using the pH 4 acetate buffer and Dragendorff's reagent. At the same time and in a similar manner, an aqueous or alcohol standard of meperidine hydrochloride containing 250 mg. per 25 ml. was assayed. When assaying injection preparations, they were spotted directly or diluted and then spotted maintaining the quantity of meperidine on the spot in the range of 100 mcg. The electrophoretic method was also compared to the conventional U. S. P. method, where applicable (4), see Table II. The upper strips in Fig. 2 il-

Table II.—Summary of Data

Compounds and Combination of Compounds	Method	Details of Method	Results: This Method, %	Results: Conventional Method, %
Meperidine, alone and in combination with other compounds	Electrophoretic	pH 4 acetate buffer (0.1 μ). Whatman No. 3 MM paper, 600 volts for 5 hr. or 250 volts for 16 hr., both at 5°. Spray is Dragendorff's reagent and colored spot of orange-red appears. (525 mμ color filter used for assay.)	96.2 98.0 97.7 95.8	95.5 98.0 98.5 95.6

Compounds and Combination of Compounds	Method	Details of Method	Sample Freshly Prepared: This Method, %	Sample Freshly Prepared: Auerbach's Method, %
Phenylephrine, alone and in combination with tetracaine	Chromatographic	Method reported by this author in This Journal, **48**, 111(1959).	111.8 110.8 102.9	109.7 110.5 104.6

Compounds and Combination of Compounds	Method	Details of Method	Samples, Subjected to Accelerated Conditions: This Method, %	Samples, Subjected to Accelerated Conditions: Auerbach's Method, %
Phenylephrine, alone and in combination with tetracaine	Chromatographic	Method reported by this author in This Journal, **48**, 111(1959).	R.T. 96.7 51° C. 86.5 65° C. 78.2 70° C. 70.6	99.1 98.6 99.0 98.3

Compounds and Combination of Compounds	Method	Details of Method	
Combination of phenylephrine, chlorpheniramine, dextromethorphan HBr, and N-acetyl-*p*-aminophenol	Electrophoretic	Buffer: pH 4 acetate (0.1 μ). Whatman No. 3 MM paper, 600 volts for 5 hr. or 150 volts for 16 hr. at 5°. Spray for chlorpheniramine and dextromethorphan is Dragendorff's and spray for N-APAP and phenylephrine is diazo reaction w/*p*-sulfanilic acid. Quantitative 525 mμ filter for chlorpheniramine and dextromethorphan, 440 mμ filter for phenylephrine, 485 mμ filter for APAP.	Only method applicable to the assay of this product containing these four compounds.

TABLE II. (*Continued*)

Compounds and Combination of Compounds	Method	Details of Method	Analysis of Solutions Containing Various Known Amounts of Hydrolyzed and Unhydrolyzed Atropine		
				—% Hydrolyzed—	
			Solution	Known Value, %	Found, %
Atropine, alone and in combination with tropine and tropic acid	Chromatographic (descending)	Reported in THIS JOURNAL, **46**, 173(1957).	1	33	34
			2	57	56
			3	67	65
			4	75	73
			5	89	90
				Chromatographic, %	Conventional Colorimetric, %
Ethinyl estradiol, alone and in combination with other drugs	Chromatographic (descending)	Solvent system: BuOH:HAc:H_2O(5:1:3) Whatman No. 1 paper, 18 hr. development. Spray reagent diazotized *p*-sulfanilic acid. Intense yellow spot assayed by photoelectric densitometry using a 440 mμ color filter.	1	93.8	96.0
			2	101.3	100.7
			3	96.8	99.1
				—Degraded Samples—	
				Chromatographic, %	Conventional Colorimetric, %
			1	54.6	98.7
			2	72.9	97.5
			3	46.7	84.7
Procaine, alone and in combination with tetracaine	Chromatographic (descending)	Solvent systems: BuOH:HAc:H_2O(5:1:3). Whatman No. 1 paper, 18 hr. development. R_f for procaine, 0.59; tetracaine, 0.80. Dragendorff's reagent for both procaine and tetracaine, orange-red spots; Diazotized *p*-sulfanilic acid for procaine, intense yellow spot.	This is a micromethod to simplify the assay of procaine and tetracaine which requires laborious extraction and correction methods. [Also requires 20-ml. sample for one method reported (9) and lately a 1-ml. sample method was also proposed (10)]. Results to be reported in detail in a subsequent paper.		

lustrate how readily meperidine can be separated from promethazine by this method. Accelerated degradation studies of this compound, alone and in combination with other drugs, can successfully be carried out by the electrophoretic procedure.

Other Applications of These Methods.—*Phenylephrine, Alone and in Combination with Tetracaine.*—A chromatographic method was used in these samples as described in a previous publication by one of the authors (1). Comparison of this method with conventional types of assays (3, 5) are indicated in Table II. For freshly prepared samples there is good agreement, but when the samples are subjected to accelerated storage conditions the chromatographic method gives the true picture of stability, since only the intact material is measured.

Atropine, Alone and in Combination with its Hydrolysis Products.—The chromatographic method was successfully applied here to study the decomposition of products containing atropine (2). The results (Table II) indicate how well the experimental values compare with the known figures.

Ethinyl Estradiol, Alone and in Combination with Other Drugs.—As the range of concentration in most preparations containing ethinyl estradiol is very low (2 to 20 mcg. per unit), it was necessary to employ extraction techniques before the paper chromatographic method could be applied. In the tablet and capsule preparations assayed by this method, carbon tetrachloride was found to be the desirable solvent, as 95 to 98% of the ethinyl estradiol was recoverable in the range of 50 to 60 mcg. Thus, a sample containing from 50 to 60 mcg. was shaken with several increments of 10 to 20 ml. of carbon tetrachloride, with the solution being decanted and filtered each time. The combined filtered solution was evaporated gently and with no heat to a volume of 10 to 20 cc. This solution was transferred to a 25-ml., pear-shaped acetylation flask. This small volume was evaporated to a very small volume (approx. 0.1 ml.) in a Rinco rotating vacuum-type evaporator. This volume was spotted on Whatman No. 1 paper with a micropipet in 0.010-cc. increments until all the solution had been spotted on the paper; using as many additional 0.1-ml. washings of carbon tetrachloride as it is felt necessary to quantitatively transfer all the material to the paper.

A reference standard is similarly treated and assayed. Descending paper partition chromatography was used with the same solvent system and color developing reagent as that used for phenylephrine (1). Ethinyl estradiol showed up as an intense yellow spot at an R_f of 0.95. (See strip *C* of Fig. 3.) These results were compared with a colorimetric method employing the extraction

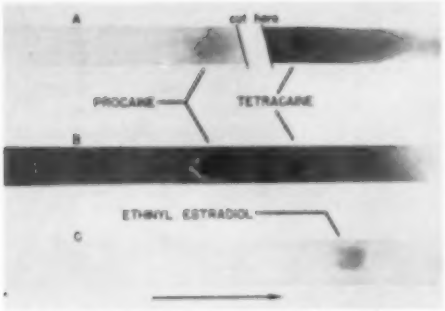

Fig. 3.—Paper chromatograms of procaine and tetracaine and ethinyl estradiol.

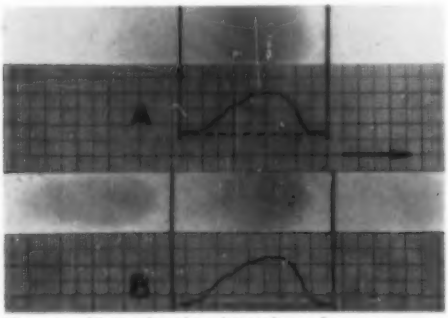

Fig. 4.—Plot of color intensity of spots *versus* position on papergrams.

procedure and colorimetric reaction of two previous publications: the ethanol-sulfuric acid color reaction of Heusghem and Jehotte (6) and a benzene extraction system as performed by Lundgren (7). These results are shown in Table II.

Procaine in Combination with Tetracaine.—A descending paper chromatographic procedure was applied for qualitatively separating procaine and tetracaine hydrochlorides. The solvent system was the same used above in the assay of ethinyl estradiol. An aqueous solution containing 20 mg. per ml. of procaine hydrochloride and 1.5 mg. per ml. of tetracaine hydrochloride was prepared. This solution was spotted on Whatman No. 1 paper in duplicate with a micropipet containing 0.010 ml., so that on the paper were two spots each of 200 mcg. of procaine hydrochloride and 15 mcg. of tetracaine hydrochloride. The paper was placed in a chromatography jar with the solvent descending the paper for eighteen hours. After the paper was dried it was cut into two strips so that different sprays could be applied to the series of spots. One strip was scanned with a short wave U. V. Mineralite lamp to locate the procaine spots, and this strip was cut so that the procaine spot could be separately sprayed with diazotized *p*-sulfanilic acid and the tetracaine with Dragendorff's reagent. The remaining strip containing both spots was sprayed with Dragendorff's reagent. The results are shown in Table II and the developed chromatograms are depicted in strips *A* and *B* of Fig. 3. The chromatographic technique described here is being used quantitatively for assay and in degradation studies of this combination of drugs. The use of the Dragendorff's reagent permits us to detect any modification on the side chain of the procaine molecule, while the diazotized reagent will detect changes on the primary aromatic amine group. Since the oral presentation of this paper, Koehler and Feldmann (8) have published a paper on the qualitative identification of local anesthetics, including procaine and tetracaine, in which they review various methods, including paper chromatography. However, as for the quantitative aspects of chromatography as applied to assay of local anesthetics after the compounds have been separated, they find elution techniques undesirable. The experiences reported in this paper and in previous publications (1, 2) indicate that satisfactory quantitation of these methods requires the use of photoelectric densitometry, thereby eliminating the need for elution.

Measurement of Total Color Density of Spots.—As stated above and in previous publications (1, 2) the total color densities of the reference and sample spots were compared by direct proportion. It was also mentioned that the total color density of each spot was measured as the total area under the curve in square millimeters. These curves were obtained by using the Photovolt densitometer model 525 containing semiautomatic plotting equipment and color filters. A typical plot of color intensity of the spots *versus* their position on the papergram is illustrated in Fig. 4. Electrophoretic strip *A* represents the migration of a reference spot of meperidine and strip *B* contains meperidine separated from a tablet preparation containing other active compounds. The procedure for this assay has been described above.

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Substituted Acetamidomalonic Esters and DL-2-Acetamido Acids as Antitumor Agents

By JOHN ANDRAKO, J. DOYLE SMITH, and WALTER H. HARTUNG

Substituted acetamidomalonic esters and DL-2-acetamido acids were prepared for the screening program of the Cancer Chemotherapy National Service Center. The synthesis of these compounds was accomplished by conventional methods. The results of screening tests performed on these compounds in the tumor systems employed by the CCNSC are given. None of the compounds on the basis of these tests appears to be a useful antitumor agent, but the results of the tests aid in establishing other approaches to the problem of cancer chemotherapy.

AMINO ACIDS and amino acid derivatives are known to function as antimetabolites (1, 2). Investigations by others have shown that among amino acid derivatives which depress the growth of Sarcoma 37 in mice are: N-iodoacetylphenylalanine (3) and N-dichloroacetyl-DL-serine (4). β-2-Thienylalanine has been reported to function as a phenylalanine antagonist in mouse Sarcoma T241 (5). The possibility that derivatives of amino acids which do not occur in nature or compounds which could potentially yield these amino acids might serve as antimetabolites in tumor systems and interfere in some way with the growth of tumor cells, prompted the preparation and examination of substituted diethyl acetamidomalonates and DL-2-acetamido acids.

The route by which these compounds were synthesized is summarized in the following equation:

$$\underset{\text{I}}{CH_3CONH{-}CH(COOC_2H_5)_2} \rightarrow \underset{\text{II}}{CH_3CONH{-}C(R)(COOC_2H_5)_2} \rightarrow \underset{\text{III}}{CH_3CONHCH(R)COOH}$$

The method used for preparing the alkylated acetamidomalonic esters (II) was essentially that reported by Snyder and co-workers (6) and by Albertson (7). The method has been reviewed recently by Cope (8).

The DL-acetamido acids were obtained by heating the alkylated acetamidomalonic esters under gentle reflux with excess sodium hydroxide. Decarboxylation was accomplished by heating the reaction mixture at pH 4. The acetamido acids precipitated when the reaction mixture was cooled and further acidified to pH 2. The procedure used for all of the compounds listed in the tables was essentially as described in the experimental section. The substituted acetamidomalonic esters which were prepared are given in Table I. The DL-2-acetamido acids are listed in Table II.

The results of screening tests performed on these compounds by the Cancer Chemotherapy National Service Center in the tumor systems, Sarcoma 180, Carcinoma 755, and Leukemia 1210 are given in Tables III and IV.

The details of typical procedures are described in the experimental section.

EXPERIMENTAL[1]

Diethyl Acetamidomalonate (I).—This intermediate was prepared according to the method described by Snyder and Smith (9).

Diethyl Acetamido-*n*-hexadecylmalonate(II).—To a solution of 2.3 Gm. (0.1 Gm.-atom) of sodium in 150 ml. of absolute ethanol in a 500-ml., 3-necked, round-bottomed flask, equipped with a stirrer and a condenser with provision for excluding moisture, there was added 21.7 Gm. (0.1 mole) of diethyl acetamidomalonate and 0.2 Gm. of potassium iodide. The solution was heated to reflux and 30.5 Gm. (0.1 mole) of *n*-hexadecyl bromide was added all at once. The mixture was refluxed and stirred for fifty hours, after which it was transferred to a 1-necked, round-bottomed flask and the ethanol removed under reduced pressure in the flash evaporator. The residue was treated with 40 ml. of water and 150 ml. of ether. The ether layer was separated and the water layer was extracted with a 50-ml. portion of ether. The combined ether extracts were evaporated on a steam bath and the residue which solidified on cooling weighed 34 Gm. (75%) and melted at 55–60°. A portion of the crude diethyl acetamido-*n*-hexadecylmalonate when recrystallized twice from petroleum ether (60–90°) melted at 64–66°.

Anal.—Calcd. for $C_{25}H_{47}NO_5$: C, 68.00; H, 10.73; N, 3.17. Found: C, 67.74; H, 10.65; N, 3.32.

In carrying out other alkylations of diethyl acetamidomalonate, the reaction mixtures were refluxed until they were neutral or almost neutral with no further change. The length of time required to

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The authors are indebted to Mrs. Venetia M. Kue for aid in carrying out the analyses.

[1] Melting points are uncorrected. Microanalyses are by Weiler and Strauss, Oxford, England, except where indicated.

TABLE I.—SUBSTITUTED DIETHYL ACETAMIDOMALONATES

$$CH_3CONH—C(COOC_2H_5)_2$$
$$\quad\quad\quad

TABLE II.—DL-2-ACETAMIDO ACIDS

$CH_3CONHCHCOOH$
|
R

R-	M. p., °C.	Yield, %	Recrystallized from	Nitrogen, % Calcd.	Nitrogen, % Found
$CH_3(CH_2)_4$-	106–107[a]	59	Ethanol-water	...	...
$CH_3(CH_2)_5$-	115–116	80	Ethanol-water	6.96	7.13[b]
$CH_3(CH_2)_6$-	108–109	82	Ethanol-water	6.51	6.32[b]
$CH_3(CH_2)_7$-	103–105[c]	84	Ethanol-water	6.11	6.37[b]
$CH_3(CH_2)_9$-	101.5–102.5[d]	76	Ethanol	5.45	5.39[b]
$CH_3(CH_2)_{10}$-	104–105	78	Ethanol-water	5.16	5.07
$CH_3(CH_2)_{11}$-	94–95.5	86	Ethanol-water	4.91	4.99
$CH_3(CH_2)_{12}$-	104–106	72	Acetone-water	4.47	4.45
$CH_3(CH_2)_{14}$-	108–109	89	Ethanol-water	4.28	4.48
$CH_3(CH_2)_{15}$-	106–108	86	Ethanol	4.10	4.17
$CH_3(CH_2)_{17}$-	108–109	62	Ethanol-water	3.79	3.92
$(CH_3)_3CCH_2CH(CH_3)(CH_2)_2$-	132.5–133.5	66	Ethanol	5.76	5.69[b]
$C_6H_5(CH_2)_2$-	147–148	80	Ethanol	6.33	6.38[b]
$C_6H_5(CH_2)_3$-	147–148	90[e]	Ethanol-water	5.95	5.98
$C_6H_5CH{=}CHCH_2$-	145–147	66	Water	6.01	5.70
o-$ClC_6H_4CH_2$-	158–159[f]	90	Ethanol-water	5.80	6.03[b]
o-$CH_3C_6H_4CH_2$-	156–157	72	Ethanol-water	6.33	6.26[b]
m-$CH_3C_6H_4CH_2$-	148–149	78	Acetone	6.33	6.31[b]
p-$CH_3C_6H_4CH_2$-	163.5–164.5	73	Ethanol	6.33	6.28[b]
1-$C_{10}H_7CH_2$-[g]	182.5–183.5	82	Ethanol	5.45	5.64[b]

[a] Lit. m. p., 106° (15). [b] Kjeldahl, Hengar method. [c] Lit. m. p., 105° (15). [d] Lit. m. p., 93–94° (16); neut. equiv.: Calcd. 257; Found: 261. [e] See Experimental. [f] Lit. m. p., 166° (17); neut. equiv.: Calcd.: 241.5; Found: 244. [g] 1-Naphthylmethyl-.

TABLE III.—SUBSTITUTED DIETHYL ACETAMIDOMALONATES SCREENING TEST RESULTS

$R{-}C(COOC_2H_5)_2$
|
$NHCOCH_3$

R-	Sarcoma 180 (T/C)[1]	Sarcoma 180 (T/C)[2]	Sarcoma 180 (T/C)[3]	Carcinoma 755 (T/C)[1]	Carcinoma 755 (T/C)[2]	Carcinoma 755 (T/C)[3]	Leukemia 1210 (T/C)[1]
$CH_3(CH_2)_4$-	0.76	...	...	0.95	...	...	0.98
$CH_3(CH_2)_5$-	0.59	...	...	1.03	...	...	1.08
$CH_3(CH_2)_6$-	0.38	1.10	...	0.96	...	...	1.02
$CH_3(CH_2)_7$-	0.73	...	...	0.256	0.51	0.97	0.82
$CH_3(CH_2)_8$-	0.93	...	...	0.97	...	...	0.82
$CH_3(CH_2)_9$-	0.53	1.0	...	0.58	...	...	0.98
$CH_3(CH_2)_{10}$-	0.63	...	...	0.97	...	...	0.85
$CH_3(CH_2)_{11}$-	0.67	...	...	0.95	...	...	0.82
$CH_3(CH_2)_{12}$-	0.52	0.40	...	0.63	...	...	0.68
$CH_3(CH_2)_{14}$-	1.26	...	...	...	...	...	...
$CH_3(CH_2)_{15}$-	0.59	...	...	0.57	...	...	1.40
$CH_3(CH_2)_{17}$-	0.67	...	...	0.43	0.69	...	0.96
$C_6H_5(CH_2)_2$-	0.90	...	...	0.526	0.86	...	1.12
o-Cl-$C_6H_4CH_2$-	0.24	0.84	...	0.82	...	...	0.95
o-$CH_3C_6H_4CH_2$-	0.41	0.64	...	1.01	...	...	0.98
m-$CH_3C_6H_4CH_2$-	0.47	0.90	...	0.65	...	...	1.22
p-$CH_3C_6H_4CH_2$-	0.185	0.48	0.84	0.81	...	...	1.02
1-$C_{10}H_7CH_2$-	0.51	0.88	...	0.89	...	...	1.04

above and was cooled and treated with a small amount of petroleum ether, a crude solid melting at 76–81° and representing a 14% yield was isolated. Recrystallization of the solid first from ether-petroleum ether and then from ethanol-water gave a white solid which melted at 86–88° and showed no depression when mixed with the product obtained above.

DL-2-Acetamido-5-phenylpentanoic Acid.—DL-2-Acetamido-5-phenyl-4-pentenoic acid, 1.00 Gm. (0.0043 mole), was dissolved in 100 ml. of absolute ethanol and 1.0 Gm. of an A-25 palladium-charcoal catalyst was added. The mixture was shaken in the Parr hydrogenation apparatus for ten minutes with an initial pressure of 50 psi. The catalyst was filtered out and washed with ethanol. The ethanol was removed from the filtrate under reduced pressure on the steam bath. The crude solid weighed 0.9 Gm. (90%) and melted at 143–146°. A sample recrystallized from ethanol-water melted at 147–148°. A mixed melting point with DL-2-acetamido-5-phenyl-4-pentenoic acid was depressed.

Anal.—Calcd. for $C_{13}H_{17}NO_3$: N, 5.95. Found: N, 5.98.

SCREENING TESTS

Tables III and IV list the results of screening tests performed by the Cancer Chemotherapy National Service Center on the tumor systems em-

TABLE IV.—DL-2-ACETAMIDO ACIDS SCREENING TEST RESULTS

R—CHCOOH NHCOCH₃ R-	Sarcoma 180 (T/C)₁	Sarcoma 180 (T/C)₂	Sarcoma 180 (T/C)₃	Carcinoma 755 (T/C)₁	Carcinoma 755 (T/C)₂	Carcinoma 755 (T/C)₃	Leukemia 1210 (T/C)₁
$CH_3(CH_2)_4$-	0.58	...	...	1.09	...	...	0.96
$CH_3(CH_2)_5$-	0.41	0.94	...	0.90	...	...	...
$CH_3(CH_2)_7$-	0.78	...	...	1.22	...	...	0.90
$CH_3(CH_2)_9$-	0.82	...	...	0.84	...	...	1.04
$CH_3(CH_2)_{10}$-	0.76	...	...	1.50	...	...	1.40
$CH_3(CH_2)_{11}$-	0.58	...	...	0.88	...	...	0.89
$CH_3(CH_2)_{12}$-	0.32	0.62	0.69	0.65	...	...	1.00
$CH_3(CH_2)_{13}$-	0.59	...	...	0.83	...	...	1.23
$CH_3(CH_2)_{17}$-	0.40	0.53	...	0.96	...	...	1.30
$(CH_3)_2CCH_2CH(CH_3)$-$(CH_2)_2$-	1.15	...	...	0.91	...	...	1.06
$C_6H_5(CH_2)_2$-	1.11	...	...	0.64	...	...	0.75
$C_6H_5(CH_2)_3$-	1.05	...	...	0.82	...	...	1.12
o-Cl-$C_6H_4CH_2$-	0.74	...	...	0.32	1.07	...	0.89
o-$CH_3C_6H_4CH_2$-	0.94	...	...	1.07	...	...	0.89
m-$CH_3C_6H_4CH_2$-	0.74	...	...	0.52	0.72	...	0.98
p-$CH_3C_6H_4CH_2$-	0.95	...	...	0.83	...	...	1.04
1-$C_{10}H_7CH_2$-	...	...	...	0.76	...	...	0.88

ployed by this agency. For Sarcoma 180 and Carcinoma 755 the designation T/C used by the CCNSC represents the "mean tumor weight of the test animals as a proportion of weight of control animals" (18). For inhibition studies if $(T/C)_1$ is less than 0.54, the substance is tested further. If the product, $(T/C)_1 \times (T/C)_2$ is less than 0.2, a third test is carried out. Should the product of $(T/C)_1 \times (T/C)_2 \times (T/C)_3$ be less than 0.08, additional tests are performed.

For Leukemia 1210, the designation T/C represents survival of test animals compared to controls. "The L-1210 tumor test is considered negative if the mean life extension of test animals beyond the fifth day of test is not increased 1.72 times that of the control animals" (18).

Of the substituted diethyl acetamidomalonates, all of the compounds in which R- was aralkyl passed at least two stages in the case of Sarcoma 180. Diethyl acetamido-*p*-methylbenzylmalonate which passed three stages in the case of this tumor system was shown in subsequent tests to be ineffective.

In Carcinoma 755, only diethyl acetamido-*n*-octylmalonate reached the third stage of screening. None of the acetamidomalonates showed any activity in L-1210.

None of the acetamido acids, except for DL-2-acetamidohexadecanoic acid in the case of Sarcoma 180, showed any activity in the screening tests. In comparing the two classes of compounds, there appears to be no correlation between their structures and the results of the screening tests.

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Growth of Medicinal Plants in Culture I

A Controlled Cabinet for Maintaining Plant Cultures

By A. E. DeMAGGIO and L. D. KING

In order to clarify the conditions necessary for obtaining maximum growth of plant cultures and maximum yields of active ingredients, a large cabinet was designed and constructed for growing cultures in a controlled environment. The cabinet, which is constructed entirely of wood, consists of two separate compartments, one above the other, each containing an illuminating system composed of a combination of fluorescent and incandescent lamps. A constant temperature is maintained in both compartments by a 1-horsepower air conditioner provided with coils for heating.

THE TECHNIQUES of plant tissue and organ culture developed during the past twenty years have provided workers in the fields of morphology, physiology, and pathology with a valuable tool for experimental studies (1, 2). The realization that whole plants, organs, tissues, and even single cells can be conveniently grown *in vitro* has produced intensified efforts to elucidate the nutritional and biochemical mechanisms of plant growth and development.

In attempting to institute a general program in culturing medicinal plants, organs, tissues, and cells for morphological and physiological investigations, the problem of obtaining the necessary space for controlled culture rooms became apparent. As a result, an investigation was undertaken to design and build a cabinet suitable for growing a variety of plant cultures under controlled conditions. The present report describes the essential details in the construction of the cabinet and directs attention to the features incorporated in the design of the apparatus which has proved indispensable for routine and experimental culture work.

DESIGN OF APPARATUS

In order to have available the maximum surface area for storing large numbers of cultures and yet keep the overall size of the cabinet at a minimum, a cabinet consisting of two separate and independent compartments, one above the other was designed. The cabinet, details of which are illustrated in Fig. 1, was constructed from $^3/_4$ in. plywood by bolting and bracing the sides and back to the roof and floor with heavy-duty angle irons. The partition separating the two compartments was inserted, bolted in place, and reinforced on all sides with metal bracings. The compartments are enclosed by double-channel sliding doors (Fig. 1) which permit easy access to the cultures while serving efficiently to contain the atmosphere within the cabinet.

Each compartment is furnished with a single 4-foot reflector type industrial fluorescent fixture modified by the addition of two incandescent sockets wired in series (Fig. 1). The fixtures are mounted directly to the roof of each compartment and light is supplied by two 40-watt T12 standard, cool white fluorescent lamps plus two 25-watt incandescent lamps. The use of fluorescent and incandescent lamps for plant growth chambers is indicated by previous studies (3, 4) which have shown the effectiveness of this combination of artificial light in increasing plant yields. The interior of the cabinet is finished in white enamel and provides highly reflective surfaces for this illuminating system. Light intensity at the level of the cultures (30 inches from the light source) is approximately 325 f.-c., as measured by a Weston master III exposure meter. The photoperiods in each compartment are regulated by an automatic timer which permits alternating light and dark cycles.

A constant temperature of 25 $\pm 2°$ is maintained in the culture cabinet by means of a thermostatically regulated 1 hp. air conditioner[1] provided with heating coils. The conditioner is mounted in the wall of the upper compartment (Fig. 1) and conditioned air from the discharge grill is channeled into the upper compartment by vertically positioned louvers and into the lower compartment by a fitted length of pipe (8 × 4 in.) adjusted to provide for maximum air dispersion. Air in the lower compartment is returned to the conditioner through another pipe (4-in. diameter) to provide efficient recycling. The completed cabinet, represented by Fig. 2, diagrammatically illustrates the direction of air flow in both compartments. Conditioned air flowing into the compartments is directed to pass

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[1] Carrier Room Weathermaker, model 51BA

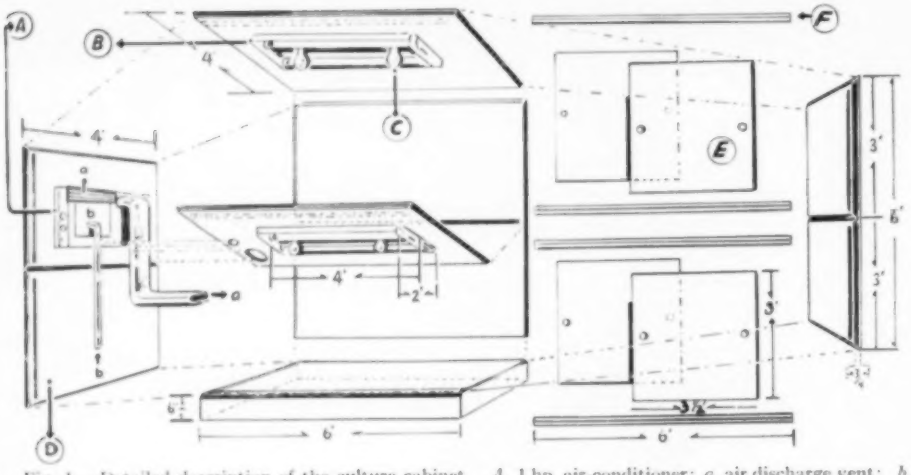

Fig. 1.—Detailed description of the culture cabinet. *A*, 1 hp. air-conditioner; *a*, air discharge vent; *b*, air return vent; *B*, lighting unit with fluorescent tubes; *C*, incandescent lamps inserted in fixture; *D*, ¾ in. plywood used throughout; *E*, sliding doors, *F*, double-channel runners for doors.

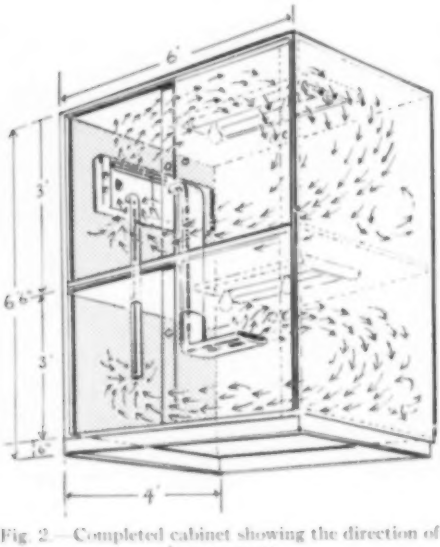

Fig. 2.—Completed cabinet showing the direction of air movement.

across the lighting units, thereby reducing the amount of heat passing from the lamps to the cultures.

In view of the lack of information regarding humidity effects on plant cultures, no stringent methods for regulating relative humidity were incorporated into the design of the cabinet. Nevertheless, the air-conditioning system presently in use is equipped to function as a dehumidifier and the relative humidity inside the cabinet is never allowed to exceed 60%.

DISCUSSION

The cabinet described in this report for the growth and maintenance of plant cultures has several advantages over the conventional culture rooms. (*a*) The cost of construction is relatively inexpensive. (*b*) A considerable amount of space for growing cultures is obtainable and yet only a small portion of the usable laboratory space is consumed. (*c*) The design of the cabinet affords the opportunity of maintaining stock cultures in one compartment and performing experiments in the other compartment by varying independently some or all of the environmental factors.

At this time it is difficult to assess the relative efficiency of the cabinet in quantitative terms. However, sufficient data have been accumulated from our studies to indicate that the environmental conditions are adequate to provide optimal growth for a variety of plant cultures (5).

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New Instrument and Method for Evaluating Tablet Fracture Resistance

By CLARENCE J. ENDICOTT, WERNER LOWENTHAL, and HERBERT M. GROSS

A new instrument and method for evaluating tablet firmness has been developed and tested. The tablet characteristic measured has been called fracture resistance. Results reported for 19 different tablets show that fracture resistance readings give smaller and more precise results than Strong Cobb hardness.

THE PHYSICAL characteristics of pharmaceutical tablets have been measured by many methods. Of various tablet properties, the one termed hardness is signally important to tablet formulation. It is the criterion which measures durability, ease of handling, and tablet firmness. This property, as most often measured today, may be more accurately described as resistance to crushing due to applied pressure. It is measured by orienting a tablet vertically on an anvil or holder and exerting increasing vertical pressure on the tablet until it crumbles or breaks. The two instruments most commonly used to measure this characteristic are the Monsanto (Stokes) and the Strong Cobb hardness testers (1–4). Comparative evaluations of the above instruments have been made by McCallum, *et al.* (5).

Other devices and methods have been improvised. A. Nutter Smith (6) used a Vickers diamond hardness tester, a "Scherographe" and a Shore "Scleroscope." K. Munzel and co-workers (7–9) used a modified Dynstat[1] to measure the bending strength of tablets. To use this instrument, specially shaped prismatic tablets of a specific size were necessary. Bending resistance, expressed in Kg./cm.^{2}, was calculated from the following equation:

$$\text{bending resistance} = 6 \times \frac{\text{bending moment}}{(\text{width} + \text{thickness})^2}$$

Various other instruments (10–17) have been used to measure what has been loosely called hardness.

In 1959 at the first annual National Industrial Pharmaceutical Research conference (18), the subject of tablet hardness was discussed by a panel on Physical Evaluation of Tablets. The consensus was that the Monsanto and Strong Cobb hardness tests indicate numbers which are meaningless and do not reflect a true measure of this physical characteristic of tablets. The panel recognized that the measurement of the force to break a tablet between the first two fingers and thumb was the most indicative, acceptable, and popular method for determining tablet firmness. The crispness of the snap and the force exerted to break the tablet measure this tablet property. This method is described in the literature by A. Nutter Smith (19).

Because of the prevailing opinion of the usefulness of the manual method, a mechanical test and method utilizing its principle were de-

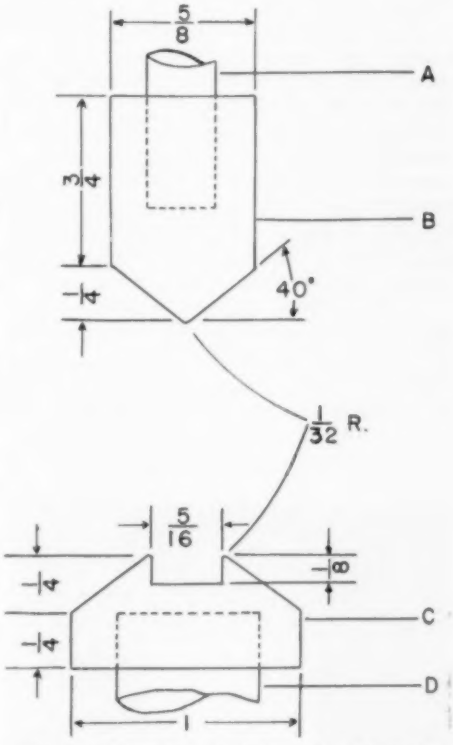

Fig. 1.—Dimensions, in inches, of adapters for Strong Cobb hardness tester. *A*, plunger on Strong Cobb apparatus; *B*, upper adapter; *C*, lower adapter which holds tablets; *D*, tablet holder on Strong Cobb apparatus.

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[1] Dynstat No. 137, made by Louis Schopper, Leipzig, Germany.

vised. K. Munzel and W. Kagi (8) and H. Spengler and A. Kaelin (16) described a method related to the one reported here. They referred to the property as a measurement of resistance to bending. The Spengler and Kaelin (16) procedure of adding mercury to provide the weight to break the tablets is not practical for rapid determinations in manufacturing areas. The Munzel and Kagi procedure requires a special tablet form which would not be the manufactured product form.

The following method modifies, with simple adapters, the Strong Cobb hardness tester. The modification consists of two auxiliary pieces. One is attached to the plunger and the other fits over the anvil of the tester. Figure 1 gives the dimensions of the two pieces. For purposes of clarity in the drawing, the two set screws are not shown. These screws keep the adapter parts in position. Figure 2 shows the front view of the actual adapters, unmounted, while Fig. 3 shows them in the conventional Strong Cobb tester.

The tablet, shown in Fig. 3, rests on two supports near its circumference. The pointed plunger rests on the tablet face and imparts continuous, increasing pressure on the center of the tablet face until it breaks (snaps) in half. The adapter parts which contact the tablet are slightly blunted to prevent a cutting action as described by Nelson (13). The fracturing pressure is provided by the pneumatic pump as in the regular operation of the tester. The property measured is described as fracture resistance. This new method mimics the manual method of testing tablet hardness or firmness but has the advantage of assigning numerical values. This method is mechanical and independent of the operator's skill, experience, and judgment.

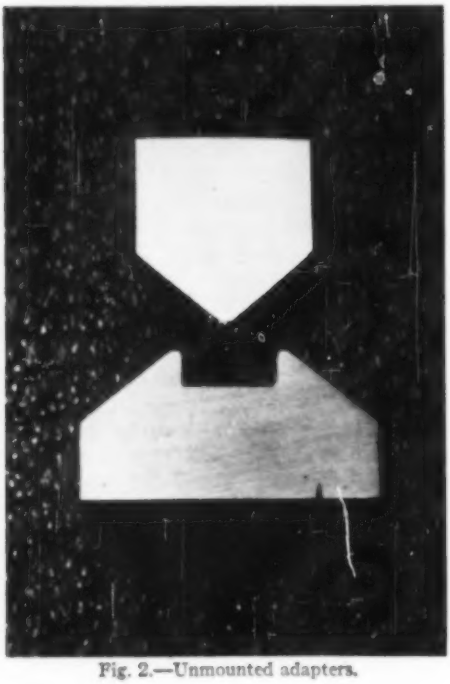

Fig. 2.—Unmounted adapters.

EXPERIMENTAL

Twelve different sized convex tablets representative of rotary tablet press production were evaluated. The composition of these tablets varied widely. In addition, seven varying sizes and shapes of placebo tablets were evaluated. The placebo tablets consisted of a mixture of lactose as filler, starch paste as the binder; and magnesium stearate, talc, and stearic acid as the lubricants. The placebo tablets were manufactured in production facilities and represent extremes in tablet size and shape. Several samples were monogrammed.

Strong Cobb hardness and fracture resistance evaluations were determined. Hardness was evaluated by the method recommended by the tester manufacturer. Fracture resistance was determined using the described adapters. Table I[1] lists data for 16 different convex tablets including the 12 production tablets and 4 convex placebo tablets. Fracture resistance *vs.* hardness for the convex tablets is plotted in Fig. 4.[1] The data for placebo tablets of various sizes and shapes are listed in Table II.[1] Fracture resistance for placebo tablets is plotted against hardness in Fig. 5.[1]

RESULTS AND DISCUSSION

Nelson (13) found that tablets were harder in the plane parallel to direction of compression. The author explained that this was due to distortion of granules which flatten during compression. There are more granules to cut in the plane parallel to the pressure. If Nelson's interpretation is correct, the apparatus described in this paper would be superior to methods now being used to measure tablet firmness since it measures resistance across the parallel plane.

Data in Tables I and II indicate that fracture resistance is less than hardness in all cases. Also, range and variance of fracture resistance readings are smaller in 18 of 19 samples. The one exception is the large capsule-shaped placebo tablet. In 9 of 19 samples, fracture resistance variance was significantly less than Strong Cobb hardness when tested using an F test (20). These facts would indicate that fracture resistance determinations, as determined in the proposed method, are more precise.

The curves obtained by the method of least squares in Figs. 4 and 5 show the relationship between the

[1] Variances in Tables I and II were calculated from the equation: variance $= [N\Sigma X_i^2 - (\Sigma X_i)^2]/N(N-1)$, where N is the number of observations, which for these data was always 10, and X is the hardness or fracture resistance readings. The curves drawn in Figs. 4 and 5 are the ones, best representing the data, going through the origin. The equation used to determine these lines is slope $= \Sigma X_i Y_i / \Sigma X_i^2$, where X represents the hardness readings and Y the fracture resistance readings.

Fig. 3.—Adapters mounted on the Strong Cobb hardness tester with tablet in place.

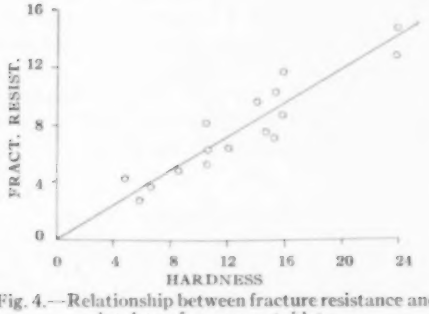

Fig. 4.—Relationship between fracture resistance and hardness for convex tablets.

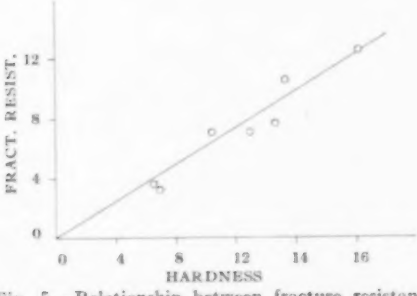

Fig. 5.—Relationship between fracture resistance and hardness for placebo tablets.

two observations. The slopes, 0.606 and 0.615, are not significantly different when applying the test for linear hypothesis for normally distributed variables.[3] This similarity of slopes would seem to indicate that this relationship is independent of tablet shape.

One of the most important advantages of the adaptation is revealed in another set of experiments designed to correlate tablet fracture resistance and thickness. In these experiments, tablets of identical composition were compressed at varying thickness on a Stokes, model BB-2 rotary tablet press. The same type of placebo granulation described before was used. Throughout the range of thickness tested, Strong Cobb hardness was maintained constant within experimental error as shown by a single classification analysis of variance. When fracture resistance of these tablets was determined, the values varied in direct proportion to tablet thickness. A single classification analysis showed the means of the four groups of fracture resistance data to be different and Duncan's multiple range test (21) showed that the fracture resistance of all four runs was different from each other. Fracture resistance increased with thickness, as expected. The data are given in Table III. This is not inconsistent with the data in Figs. 4 and 5 because in this last experiment, thickness was the only independent variable, whereas in the previous experiments, there were several uncontrolled independent variables. The slope of the line is 1.213. With this information, tablet thickness can be predetermined by specifying the fracture resistance required.

This difference is extremely significant since it provides a reliable means of showing that tablet firmness is related to tablet thickness and that tablets of increased thickness will be more resistant to breakage in routine handling, bottling, shipping, and dispensing. While this physical property would be expected, it has not been previously measured and a practical measuring device has not been described.

The more precise, smaller readings obtained for fracture resistance are also advantageous. A 0 to 30-pound scale on a $2^1/_2$-in. diameter pressure gauge could replace the present gauge on the tester. This would further increase the accuracy of the readings. The lower part of the device which holds the tablets, as described in Figs. 1, 2, and 3, also can be modified with a thumb screw arrangement so that the two supports of the tablet become adjustable. The supports can then be moved to accommodate tablets of various size, thus providing versatility. Additional research is required to derive a design which will accommodate all tablet sizes, which can be calibrated for reproducible determinations, and

[3] The F test used to determine whether the slopes were significantly different was $SS_1 = \Sigma Y^2 - b(\Sigma XY)$, where SS_1 is the sum of squares for convex tablets, Y is the fracture resistance readings, X is the hardness readings, and b is the slope for the convex tablets. $SS_2 = \Sigma Y^2 - b\ (\Sigma XY)$, where SS_2 is the sum of squares for placebo tablets, and Y, X, and b have the same meanings as above. Only data for placebo tablets are used. Combining data for all the tablets, $b = \Sigma XY/\Sigma X^2$ = pooled slope and $SS_t = \Sigma Y^2 - b\ (\Sigma XY)$, is the total sum of squares.

Analysis of Variance

Source	$d.f.$	SS	MS	F
Between slopes	1	$SS_t - SS_1 - SS_2$	SS/df	$\frac{MS \text{ (between slopes)}}{MS \text{ (about indiv. lines)}}$
About individual lines	$N_1 + N_2 - 4$	$SS_1 + SS_2$	SS/df	1

Table I.—Hardness and Fracture Resistance Evaluation for Convex Production Tablets

Tablet	Diam., in.	Thickness, in.	Hardness[a]	Hardness Range	Hardness Variance	Fract. Resist.[a]	Fracture Resistance— Range	Fracture Resistance— Variance
1	13/32	0.169	5.8	4.0	1.883	2.8	0.7	0.0388[b]
2	1/2	0.219	23.8	8.8	11.121	15.1	4.7	3.673
3	1/2	0.243	23.7	9.4	8.190	13.1	5.2	3.052
4	10/32	0.180	10.6	4.2	1.201	6.4	1.6	0.244[b]
5	1/2	0.240	15.9	4.8	2.852	11.9	2.8	1.043
6	1/2	0.200	15.2	4.6	1.867	7.3	1.8	0.296[b]
7	13/32	0.180	8.5	2.1	0.456	4.8	1.5	0.198
8	15/32	0.231	10.5	6.4	4.069	5.3	2.2	0.513[b]
9	10/32	0.168	4.8	2.4	0.524	4.2	0.6	0.0640[b]
10	1/2	0.181	15.8	1.9	0.391	8.9	2.2	0.297
11	1/2	0.189	14.6	7.4	8.335	7.7	6.0	4.200
12	1/2	0.224	15.3	4.9	2.765	10.5	3.8	1.729
13	9/16	0.159	6.5	6.8	7.336	3.7	2.7	0.943[b]
14	1/2	0.221	10.4	7.4	8.810	8.2	7.1	5.344
15	3/4	0.175	12.0	4.2	1.458	6.5	2.0	0.384[b]
16	1/2	0.225	14.0	8.3	9.387	9.8	2.9	0.765

[a] Average of 10 readings. [b] Significant F test at 5% level, where F = hardness variance/fracture resistance variance, and is significant if greater than 3.18 (20).

Table II.—Hardness and Fracture Resistance Evaluation for Placebo Tablets

Tablet	Diam., in.	Thickness, in.	Hardness[a]	Hardness Range	Hardness Variance	Fract. Resist.[a]	Fracture Resistance Range	Fracture Resistance Variance
Capsule shape	...	0.245	12.9	3.0	0.737	7.1	3.7	1.240
Large football shape	...	0.227	20.1	7.5	6.160	12.5	3.2	1.016[b]
Convex	1/2	0.180	14.6	7.4	8.335	7.7	6.0	4.200
Small football shape	...	0.185	6.9	2.3	0.598	3.2	1.9	0.358
Convex	1/2	0.224	15.3	4.9	2.765	10.5	3.8	1.729
Convex	9/16	0.159	6.5	6.8	7.336	3.7	2.7	0.943[b]
Convex	1/2	0.221	10.4	7.4	8.810	8.2	7.1	5.344

[a] Average of 10 readings. [b] Significant F test at 5% level, where F = hardness variance/fracture resistance variance, and is significant if greater than 3.18 (20).

Table III.—Relationship Between Tablet Fracture Resistance and Thickness[a]

Run	Thickness, mm.	Strong Cobb Hardness	Fracture Resistance
1	3.685	6.1	3.1
2	4.504	6.2	4.3
3	5.092	5.8	4.8
4	5.650	6.2	5.4

[a] Average of 10 determinations.

which will facilitate the acceptance of the tablet fracture resistance concept.

The instrument and method described in this article should replace the manual method now so widely used and recognized. The new method can be readily used by research, manufacturing, and control. It represents an addition to the scientific technology in tablet manufacture.

CONCLUSIONS

1. A new instrument and method for evaluating tablet firmness has been developed and tested. The tablet characteristic measured has been called fracture resistance.

2. The new method simulates the now widely used and recognized manual tablet breakage test.

3. The new instrument can be readily adapted to the Strong Cobb hardness tester.

4. Differences in fracture resistance as related to tablet thickness are detectable on this instrument. This is of importance to the manufacturer and pharmacist in preventing excessive losses due to tablet breakage.

5. Results reported for 19 different tablets show that fracture resistance readings give smaller and more precise results than Strong Cobb hardness.

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Qualitative and Quantitative Tests for Isobucaine Hydrochloride

By EDWARD G. FELDMANN†, JOHN J. HEFFERREN, HENRY M. KOEHLER, and JULIAN R. REASENBERG‡

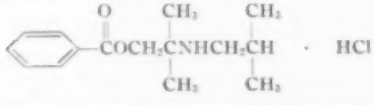
A series of qualitative tests and quantitative assay procedures are presented for isobucaine hydrochloride. These data result from a collaborative laboratory study by research personnel of the drug firm and the American Dental Association. The methods chosen are discussed, their particular applications noted, and their limitations described.

ISOBUCAINE HYDROCHLORIDE,[1] is an unsubstituted benzoic acid ester of the secondary aminoalcohol, 2-isobutylamino-2-methylpropanol. It is similar in structure to meprylcaine hydrochloride,[2] which has a *n*-propyl substituent on the amino group. Isobucaine, like procaine, is an ester-type local anesthetic which is hydrolyzed in the blood (1). It may be classified as a moderately long acting anesthetic agent.

The chemical tests have been developed to provide data on those drugs appearing in "Accepted Dental Remedies." The limits set for these tests seem to be reasonable with expected normal analytical and manufacturing variation for isobucaine hydrochloride and its preparations. It is to be expected that there may be or will be dosage forms of isobucaine hydrochloride where these tests will not apply without appropriate modifications.

ISOBUCAINE HYDROCHLORIDE

2-Isobutylamino-2-methylpropyl benzoate hydrochloride; $C_{15}H_{23}NO_2 \cdot HCl$; M.W. 285.82. The structural formula of isobucaine hydrochloride may be represented as follows:



Physical Properties.—Isobucaine hydrochloride is a white, odorless, crystalline solid, m. p. 183–185°, U. S. P. class I. It is freely soluble in water, alcohol, and chloroform, and very slightly soluble in ether. Approximately 1 Gm. of isobucaine hydrochloride will dissolve in 100 ml. of isopropanol at 25°. The pH of a 2% aqueous solution is about 5.9.

Identity Tests.—Dissolve about 100 mg. of isobucaine hydrochloride in 10 ml. of water and add 2 drops of nitric acid followed by 1 ml. of silver nitrate T. S.; a white precipitate forms which is insoluble in diluted nitric acid, but the precipitate is soluble in diluted ammonia solution (presence of chloride).

Dissolve about 100 mg. of isobucaine hydrochloride in 10 ml. of water, heat almost to boiling, and add, with stirring, 1 ml. of a saturated solution of picric acid in 20% ethanol. Allow the solution to cool slowly and collect the precipitate on a filter. Wash the precipitate with a few small portions of water and dry in a vacuum desiccator over magnesium perchlorate for eighteen hours. The picrate melts with decomposition at 179–180°, U. S. P. class I; the picrate may be recrystallized from 50% ethanol. (Caution! Picrates may be explosive.)

Dissolve about 200 mg. of isobucaine hydrochloride in 10 ml. of citric acid buffer, pH 6, and add, with stirring, 100 ml. of an 0.6% aqueous solution of sodium tetraphenylboron. Continue occasional shaking for about two minutes and then allow the turbid solution to stand overnight. Collect the white crystalline precipitate on a sintered-glass filter of medium porosity. Wash the precipitate with a few small portions of water and dry it in a vacuum desiccator over magnesium perchlorate for twenty-four hours. The tetraphenylborate derivative melts with decomposition at 141–144°, U. S. P. class I; the melting point may be sharpened by recrystallization from a mixture of methanol and water.

Dissolve about 100 mg. of isobucaine hydrochloride in 10 ml. of diluted hydrochloric acid, cool to 0° in an ice bath, and add 5 ml. of 20% sodium nitrite solution with gentle stirring. Add about 2 ml. of a solution of 100 mg. β-naphthol in 5 ml. of 5% sodium hydroxide. The solution rapidly turns green in color, and no bright orange-red precipitate is formed (absence of primary aminophenyl group).

[Preparation of azobenzene-4-diazonium fluoborate.—Dissolve 4.9 Gm. of 4-aminoazobenzene (*p*-phenylazoaniline) in a minimum volume of dimethylformamide and add 15 ml. concentrated hydro-

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[1] The trade name for isobucaine hydrochloride is Kincaine Hydrochloride.
[2] The trade name for meprylcaine hydrochloride is Oracaine Hydrochloride.

chloric acid. While keeping the mixture between 0–5° in an ice bath, add dropwise, with stirring, a solution of 2 Gm. sodium nitrite dissolved in a minimum volume of water. Stir the reaction mixture for ten minutes, add 250 mg. of urea to neutralize the excess nitrous acid, and suction-filter the cold mixture. To the filtrate add a solution of 3.1 Gm. of sodium tetrafluoroboron, dissolved in a minimum volume of water. Collect on a sintered-glass filter of medium porosity the solid precipitate which forms, and wash thoroughly with small portions of water, methanol, and ether. Dry the precipitate in a vacuum desiccator over magnesium perchlorate for twenty-four hours. The azobenzene-4-diazonium fluoborate so prepared can be preserved in a tightly stoppered glass bottle for some time.]

Dissolve 100 mg. of isobucaine hydrochloride in 10 ml. dimethylsulfoxide. Prepare a 0.25% solution of azobenzene-4-diazonium fluoborate in dimethyl sulfoxide. Add two drops of the diazonium fluoborate reagent to two drops of the isobucaine hydrochloride solution on a white spot plate. Wait five minutes, then add two drops of 10% tetraethylammonium hydroxide solution. Note immediately the formation of red globules, which tend to lose their color and fade. The formation of the red color distinguishes isobucaine hydrochloride from meprylcaine hydrochloride (no color) and procaine hydrochloride (deep purple color).

Absorption Characteristics.—An 0.01% solution of isobucaine hydrochloride in water exhibits an ultraviolet absorbance maximum at about 275 mμ [absorptivity (1%, 1 cm.) about 33.5] and a minimum at about 260 mμ. An 0.001% solution in water exhibits an absorbance maximum at about 232 mμ [absorptivity (1%, 1 cm.) about 443] and a minimum at about 208 mμ. An 0.001% solution of isobucaine base in 2,2,4-trimethylpentane exhibits an absorbance maximum at about 227 mμ [absorptivity (1%, 1 cm.) in terms of the hydrochloride salt, about 451] and a minimum at about 208 mμ.

The infrared absorption spectrum of a 0.3% mixture of isobucaine hydrochloride in potassium bromide, in a disk of about 0.83 mm. thickness, is shown in Fig. 1.

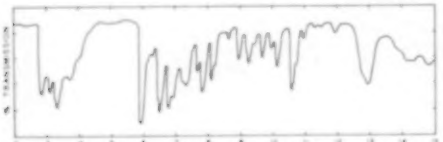

Fig. 1.—Infrared absorption of isobucaine hydrochloride.

Purity Tests.—Dry about 1 Gm. of isobucaine hydrochloride, accurately weighed, at 105° for four hours; the loss in weight does not exceed 0.5%.

Char about 1 Gm. of isobucaine hydrochloride, accurately weighed, cool the residue, add 1 ml. of concentrated sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh. The residue does not exceed 0.1%.

Assay.—Transfer to a 200-ml. beaker about 400 mg. of isobucaine hydrochloride, accurately weighed. Add 50 ml. of glacial acetic acid and 10 ml. of 6% mercuric acetate in glacial acetic acid. Titrate potentiometrically with 0.1 *N* perchloric acid in glacial acetic acid, or alternately titrate to an aqua end point, using one drop of 0.2% crystal violet in glacial acetic acid as an indicator. Each milliliter of 0.1 *N* perchloric acid is equivalent to 28.58 mg. of isobucaine hydrochloride. The amount of isobucaine hydrochloride, $C_{18}H_{29}NO_2 \cdot HCl$, is not less than 98.0% nor more than 102.0%.

ISOBUCAINE HYDROCHLORIDE AND EPINEPHRINE INJECTION

Identity Tests.—The solution responds to the identity tests, except the azobenzene-4-diazonium fluoborate color test, in the monograph for isobucaine hydrochloride.

Assay.—*Isobucaine Hydrochloride.*—Transfer to a 60-ml. separator an amount of solution, accurately measured, equivalent to about 200 mg. of isobucaine hydrochloride, and add exactly 10 ml. of spectro grade or redistilled practical grade 2,2,4-trimethylpentane. Water, saliva, or other lubricant which is not soluble in trimethylpentane should be used as a separator stopcock lubricant. Add 1 ml. of sodium hydroxide T. S., immediately stopper the separator, and shake vigorously for one minute. Allow the layers to separate and withdraw the lower (aqueous) layer completely (loss of part of the organic layer is not important). Filter the remainder of the organic layer through qualitative filter paper to remove any traces of the aqueous phase. Transfer a 5-ml. aliquot of the filtered trimethylpentane fraction to a 100-ml. volumetric flask, fill to the mark with trimethylpentane, and mix. Transfer 5 ml. of this solution to a 50-ml. volumetric flask, fill to the mark with trimethylpentane, and mix. Again, transfer a 5-ml. aliquot of this solution to a 50-ml. volumetric flask, fill to the mark with trimethylpentane, and mix. Similarly, prepare a reference solution using isobucaine hydrochloride of a purity corresponding to the tests previously described for isobucaine hydrochloride active ingredient. Spectrophotometrically determine the absorbances of the sample solution and of the reference solution in 1-cm. quartz cells at the maximum at about 227 mμ, using the same grade of trimethylpentane as a blank. The concentration of anhydrous isobucaine hydrochloride in the solution in mg./ml. = (absorbance of sample × concentration of the reference solution) ÷ (absorbance of the reference solution). The amount of isobucaine hydrochloride is not less than 95.0% nor more than 105.0% of the labeled amount.

Transfer to a 60-ml. separator an amount of solution, accurately measured, equivalent to about 40 mg. of isobucaine hydrochloride. Add 8 ml. of water and 1 ml. of sodium hydroxide T. S. and extract the mixture with three 10-ml. portions of chloroform. Wash the combined chloroform extracts with 10 ml. of water. Filter the chloroform extracts through a tight cotton plug, using additional chloroform to complete the transfer. Titrate the chloroform solution colorimetrically to a red end point with 0.01 *N* perchloric acid in dioxane using 2 drops of 0.1% methyl red in methanol as an indicator. Alternately, add sufficient acetonitrile to the combined, washed chloroform extracts to give a chloroform-acetonitrile ratio of about 4 to 1 and titrate potentiometrically. Each milliliter of 0.01 *N*

perchloric acid is equivalent to 2.858 mg. of isobucaine hydrochloride. The amount of isobucaine hydrochloride is not less than 95.0%, nor more than 105.0% of the labeled amount.

Epinephrine.—The concentration of epinephrine may be determined, using the U. S. P. colorimetric assay procedure (U. S. P. XVI, p. 895). The amount of epinephrine is not less than 95.0%, nor more than 110.0% of the labeled amount.

DISCUSSION

U. S. P. terminology for solubility, melting points, and reagents have been used wherever feasible.

Identity Tests.—A number of identity tests have been included so that this data would be available for comparison with similar tests for other local anesthetic agents (2). The paper chromatographic R_f values for isobucaine hydrochloride in the hydrochloric acid and acetic acid systems described by Koehler and Feldmann (2) are 0.82 and 0.86, respectively.

While azobenzene-4-diazonium fluoborate (3) is not commonly available and its solution in dimethylsulfoxide should not be stored for prolonged periods of time, this is a useful colorimetric method to distinguish between isobucaine and meprylcaine hydrochlorides. The azobenzene-4-diazonium fluoborate color reaction must be carried out in dimethylsulfoxide, hence this test cannot be applied to the usual injectable solutions until the isobucaine has been extracted and is available as a dry solid.

Quantitative Methods.—The active ingredient, isobucaine hydrochloride, can be determined by the usual Kjeldahl nitrogen method which is accurate but somewhat involved. The volumetric chloride ion methods are rapid and accurate; however, such methods determine the inactive portion of the molecule, and are subject to serious interferences especially when indiscriminately applied to dosage forms of unknown composition.

The direct titration of isobucaine hydrochloride in glacial acetic acid with perchloric acid as a titrant and mercuric acetate as a halide-binding agent is a method which is rapid and accurate. As illustrated in curve *B* of Fig. 2, this titration can easily be followed colorimetrically with crystal violet indicator, or potentiometrically with usual glass-calomel electrodes, or the Beckman standard combination electrode (4). Analysis of a commercial sample of isobucaine hydrochloride using the colorimetric perchloric acid titration procedure gave an average value of 99.2% with a standard deviation of 0.2%.

The parenteral dosage forms of isobucaine hydrochloride normally contain a vasoconstricting agent such as epinephrine. When the isobucaine hydrochloride is present in these solutions at the 2% level, a concentration of vasoconstrictor of the order of 1:50,000 does not contribute significant interference to the usual methods of assay. However, by utilizing a dilute solution of sodium hydroxide to convert isobucaine hydrochloride in solution to its water-insoluble free base, the phenolic vasoconstrictor as well as any acidic decomposition products are converted to their corresponding water-soluble sodium salts. Thus it is possible to separate selectively the isobucaine into an organic solvent where it can be then titrated or determined spectrophotometrically.

To determine the extent of simultaneous hydrolysis occurring during the extraction of the isobucaine base, a solution of isobucaine hydrochloride was made basic with sodium hydroxide T. S., and the resultant mixture was shaken in a separator with 2,2,4-trimethylpentane over a period of ten minutes. Aliquots were periodically taken from the trimethylpentane phase and the ultraviolet absorbance of each aliquot determined on a recording spectrophotometer. No significant change in the intensity or location of the ultraviolet absorption bands was found, indicating that there is no significant hydrolysis of the isobucaine during this extraction step.

The water-insoluble isobucaine base can be extracted and determined by a method similar to the U. S. P. XVI method for procaine hydrochloride injection, subsequent to removal of the extracting solvent (5). The base is dissolved in a water-alcohol medium and titration proceeds with 0.1 *N* hydrochloric acid as the titrant. A more direct procedure is the titration of the isobucaine in the extraction solvent such as chloroform with 0.01 *N* perchloric acid in dioxane as a titrant and with methyl red as an indicator. This titration can be followed potentiometrically more effectively by the addition of acetonitrile which lowers the resistance of the titration medium; see curve *A* of Fig. 2. The calomel-glass combination or the Beckman combination

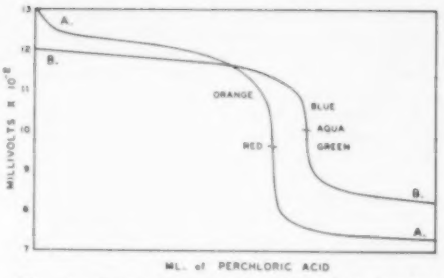

Fig. 2.—Potentiometric titration of isobucaine base in a 4:1 mixture of chloroform-acetonitrile with 0.01 *N* perchloric acid in dioxane as a titrant and methyl red as an indicator, curve *A*. Potentiometric titration of isobucaine hydrochloride in glacial acetic acid with 0.1 *N* perchloric acid in glacial acetic acid as a titrant and with mercuric acetate and crystal violet indicator added, curve *B*.

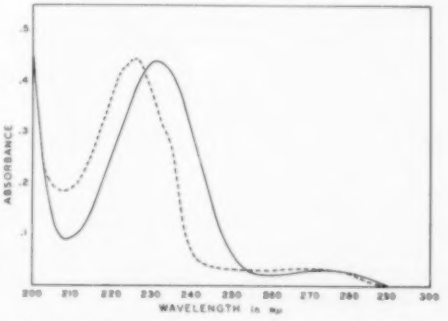

Fig. 3.—Ultraviolet absorption of isobucaine hydrochloride in water——, and of isobucaine base in 2,2,4-trimethylpentane - - -.

electrode can be used with the solvent combination and titrant (4). The recovery of isobucaine by this perchloric acid method from a synthetic 2% solution of isobucaine hydrochloride was 99.4% with a standard deviation of 0.7%.

By utilizing 2,2,4-trimethylpentane as an extracting solvent, the isobucaine base can be determined spectrophotometrically in this solvent after appropriate dilutions (Fig. 3). Trimethylpentane boils at 98° so possible errors due to evaporation are not a problem. Since the rather low ultraviolet wavelength of 227 mμ is used for assay purposes, it is preferable that all dilutions and the blanks be made from the same sample of spectro or redistilled practical grade trimethylpentane. Analysis of isobucaine hydrochloride in a 2% synthetic or standard solution by the ultraviolet method gave an average value of 99.6% with a standard deviation of 0.7%. Thus, this method compares favorably with the perchloric acid titrimetric method, and both methods appear to possess good accuracy and precision.

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Properties and Analysis of Haloperidol and Its Dosage Forms

By PAUL J. A. W. DEMOEN

Haloperidol (R 1625), a new synthetic organic compound belongs to a group of basic butyrophenones which constitute a distinct new class of neuroleptic substances. It occurs as a white, amorphous, or microcrystalline solid. It is only slightly soluble in water but can be dissolved in the presence of lactic or tartaric acid, giving stable solutions. Haloperidol may be formulated as multiple-dose solution for oral administration, as solution for injection, or tablets. It can be determined in its dosage forms by common analytical procedures. Haloperidol is stable in its formulations over long periods of time.

ON SYNTHESIZING a series of over 1,000 new substituted piperidines, it was found that the basic butyrophenones therein had powerful C.N.S.-depressant activity in various species (1). Among them 4′-fluoro-4-[1-[4-hydroxy-4-(4′-chlorophenyl)-piperidine]]-butyrophenone, generic name haloperidol, was selected for further pharmacological study on mice, rats, cats, dogs, and monkeys (2–10). The pharmacological results showed haloperidol to be a strong C.N.S. depressant with activity ratios of 3 to 200 on a weight basis, as compared to chlorpromazine, under various experimental conditions. Due to the specific properties of haloperidol and of the other butyrophenones, they should be considered as a distinct new class of neuroleptic agents (1, 3, 7, 8).

The clinical reports confirmed the pharmacological results. They designate haloperidol as a major neuroleptic agent (11–18) in the treatment of agitation of any etiology (13, 14, 19, 20, 57).

Among the side effects of haloperidol, extrapyramidal symptoms (17, 21–25), and parkinson-like reactions (13, 26) were observed.

Beside the specific neuroleptic properties, the antiemetic action of haloperidol in cases of chronic vomiting should be mentioned (19, 27).

In combination with analgesics, haloperidol is used successfully in surgery for producing a state of "neuroleptanalgesia" (28–30).

Although its toxicity is low as compared to its activity (11, 12, 24, 31–34), the individual sensitivity against haloperidol is variable (19, 27, 35). Aged persons seem to be especially sensitive and tend to show side effects at lower dose levels (22, 36).

Haloperidol can be given orally with the same effectiveness as the injections. Therefore, the most preferred and most used dosage form is the multiple-dose solution for oral administration (12–14, 17, 18, 22–24, 31, 34, 37–39), while intramuscular or intravenous injections (11, 14, 31, 34, 37, 39), and tablets (31) are also used.

The most frequently used daily dose ranges from 0.5 to 10 mg. b. i. d. or t. i. d. (11, 12, 16–18, 20–27, 34, 37–41), whereas two 0.5 mg. doses a day seem to be effective as an antiemetic (19, 27).

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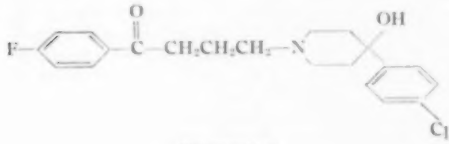
PHYSICAL PROPERTIES

Haloperidol is a white to faintly grayish, amorphous or microcrystalline powder, melting point 147–149°. It is stable to the air, but discolorizes slowly under the influence of light. Its empirical formula is $C_{21}H_{23}ClFNO_2$, mol. wt. 375.86. It may be represented structurally as follows:



Haloperidol

It forms a stable hydrochloride salt, m. p. 226–227.5°. Haloperidol is practically insoluble in water, diluted mineral acids, solutions of alkali hydroxides, and ammonia water. In the presence of 1% lactic acid or tartaric acid, stable solutions can be prepared containing up to 20 mg. of the substance per ml. One gram of it dissolves at room temperature in about 15 ml. of chloroform, 50 ml. of acetone, 55 ml. of methanol, ethanol, or ethyl acetate, 90 ml. of benzene, 200 ml. of ether or isopropyl alcohol, or 220 ml. of hexane. Haloperidol is a weak monovalent base with pK'a = 8.25 when measured in 50% methanol, as described by A. H. Beckett, *et al.* (42).

When measured at a concentration of about 1.5 mg.% in isopropyl alcohol containing 10% v/v of 0.1 *N* hydrochloric acid (43), haloperidol shows two ultraviolet absorption bands with maxima at 220 mμ, molar absorptivity (ε) about 15,000, and at 244 mμ, ε-value about 12,800. Minimum absorption is found at 231 mμ, ε-value about 7,800. The infared spectrum (2.5 to 15 μ) of about one mg. of haloperidol in a potassium bromide disk shows intense absorption maxima at the following wavelengths: 3.2 μ, OH-bonding; 3.4 to 3.6 μ, CH-stretching; 6.0 μ, carbonyl stretching of the ketone; 6.3 μ and 6.8 μ, aromatic CH; 8.2 μ, C—OH stretching or deformation in tertiary alcohol; 8.6 μ to 11 μ, several deformation modes, ring vibrations, and C F stretching; 12.0 μ, CH out-of-plane bending deformation in *para*-disubstituted benzene.

Haloperidol forms colored complexes with different color indicators (bromocresol purple, bromocresol green, methyl orange), analogous to the complexes formed by many alkaloids and synthetic basic drugs (44–50). These complexes which have a high color intensity can be extracted quantitatively from aqueous solutions by means of an organic solvent. Using this method, relatively small (≤25 mcg.) amounts of the product can be determined colorimetrically. With methyl orange as the indicator and benzene as the extracting agent, this method has been used successfully in this laboratory to determine the amount of excreted haloperidol in the urine and feces of laboratory animals (51). After extraction from biological material by an appropriate procedure and subsequent separation by paper chromatography using the ascending technique of Curry (52), haloperidol could be determined (51) with sufficient accuracy as follows: The piece of paper bearing the chromatographed haloperidol is cut out and burned in an oxygen atmosphere as described by W. Schöniger (53, 54). The fluoride thus formed is then determined colorimetrically using the method of Singer and Armstrong (55). Haloperidol gives an orange-brown spot, when sprayed with the modified Dragendorff reagent (56), after paper chromatography on buffered Whatman No. 1 paper according to Curry (52). Furthermore, it gives a fluorescent spot when viewed under an ultraviolet lamp, R_f ±0.75 (methanol:amyl alcohol : benzene : water, 40:20:40:5).

TYPICAL FORMULATIONS

Multiple-Dose Solution for Oral Administration.—Solutions for oral administration containing 2 mg. of haloperidol per ml. were prepared by dissolving the required amount of the substance in purified water in the presence of a slight excess of lactic acid. The solution should be protected from sunlight or bright daylight. Methylparaben (1.9 mg. per ml.) is added as a preservative. The solution is filtered and stored in amber glass vials; pH of the solution is 3.3.

Injection.—Solutions for intramuscular or intravenous injection containing 5 mg. of haloperidol per ml. were prepared by dissolving the required amount of the product in water for injection in the presence of 5 mg. of lactic acid solution per ml. As a preservative, 0.5 mg. of methylparaben and 0.05 mg. of propylparaben per ml. are added. The solution is sterilized by filtration and stored in 1-ml. amber glass ampuls. The pH of the solution is 3.2. The solution is slightly hypotonic (−0.45°).

Compressed Tablets.—Tablets with a total weight of 100 mg. containing 1 mg. of haloperidol, lactose, sucrose, and corn starch were prepared by wet granulating, having a disintegration time of one and one-half to two and one-half minutes at 37° in artificial gastric juice, and a hardness of about 3 Kg. (Stokes hardness tester).

Stability. The stability of haloperidol in its different formulations was followed at various storage temperatures over relatively long periods of time. The results are summarized in Tables I, II, and III. They indicate that haloperidol is stable in its various dosage forms. As a result of its twofold halogen substitution however, the product is sensitive to light. When exposed to sunlight or bright daylight, solutions of haloperidol in clear glass containers discolor after a few hours, and give a grayish-red precipitate after several weeks. When stored in amber glass containers, no discoloration or precipitate whatever could be observed, even after storage periods of up to eighteen months.

TABLE I.—STABILITY OF HALOPERIDOL IN SOLUTIONS FOR ORAL ADMINISTRATION, 2 MG. PER ML.

Time, mo.	Per Cent Stability at Storage Temperature 60°	45°	22 ± 2°	4 ± 1°
0	100	100	100	100
1	102	100	100	97
2	99	101	101	100
4	97	97	100	99
6	98	99	98	98
9	..	100	101	101
12	..	102	99	98
18	..	100	98	97

TABLE II.—STABILITY OF HALOPERIDOL IN SOLUTIONS FOR INJECTION, 5 MG. PER ML.

Time, mo.	Per Cent Stability at Storage Temperature 60°	45°	22 ± 2°	4 ± 1°
0	100	100	100	100
1	100	99	100	98
2	101	98	98	99
3	99	97	99	99
4	97	98	102	97
6	99	100	99	100
9	...	97	97	102
12	...	99	100	100
18	...	98	97	101

TABLE III.—STABILITY OF HALOPERIDOL IN COMPRESSED TABLETS, 1 MG. PER TABLET

Time, mo.	Per Cent Stability at Storage Temperature 60°	45°	22 ± 2°
2	100	100	100
3	104	103	97
6	100	101	99
9	98	97	102
12	99	98	98
18	96	97	100

ANALYTICAL PROCEDURES

Haloperidol can be determined quantitatively in its formulations by ultraviolet absorption spectrometry or by acidimetric titration in nonaqueous medium. Because it has a typical absorption pattern and a high extinction coefficient, the measurement of its U. V. spectrum presents a specific and sensitive method of analysis. In the presence of parabens which are used as preservatives in haloperidol solutions, the U. V. spectrum can be measured only after extraction of these interfering substances from aqueous acidified solution. It has been found that haloperidol quantitatively remains in the aqueous layer, whereas the parabens are extracted completely with ether.

Solution for Oral Administration, 2 mg. per ml.—*U. V. Spectrum.*—A 2.5-ml. quantity (5 mg. of haloperidol) of the solution is transferred to a 50-ml. separatory funnel, diluted with 2.5 ml. water, and acidified with 5 ml. 1 *N* hydrochloric acid. The methylparaben is extracted from this solution by shaking with 20-, 20-, 10-, and 10-ml. portions of ether. The combined ether extracts are washed with a 5-ml. portion of water which is added to the acid layer. The main aqueous solution is transferred to a 50-ml. volumetric flask and diluted to the mark with water. Ten milliliters of this solution is pipetted into a 100-ml. volumetric flask and diluted to the mark with isopropyl alcohol. The absorption of the dilution is measured against isopropyl alcohol containing 10% v/v of 0.1 *N* hydrochloric acid. Maximum absorption is found at 220 mμ and 244 mμ. From the absorbance at 244 mμ, the concentration of haloperidol is calculated, using a molar extinction coefficient (ϵ) of 12,800.

Titration.—A 20-ml. portion (40 mg. of haloperidol) of the solution is pipetted into a 150-ml. beaker and evaporated on a boiling water bath to just dryness. The residue is dissolved by gentle warming in about 30 ml. glacial acetic acid and cooled. After the addition of 0.2 ml. of a 1% solution of α-naphtholbenzein in glacial acetic acid as an indicator, the solution is titrated with 0.02 *N* perchloric acid in the same solvent. One milliliter of 0.02 *N* perchloric acid corresponds to 7.5172 mg. of haloperidol.

Solution for Injection, 5 mg. per ml.—Haloperidol is determined in the solution for injection by the same methods as described above, using 1 ml. for the ultraviolet spectrometry and 10 ml. for the nonaqueous titration.

Compressed Tablets, 1 mg. per tablet.—An amount of finely powdered tablets, corresponding to 5 mg. of haloperidol, is transferred to a 100-ml. volumetric flask. About 60 ml. of isopropyl alcohol is added, the mixture warmed to 50°, and shaken for fifteen minutes. After cooling, isopropyl alcohol is added to volume, the contents of the flask are mixed, and filtered through a retentive paper filter. The first few ml. of the filtrate is discarded. Five milliliters of the filtrate is pipetted into a 50-ml. volumetric flask, 5 ml. of 0.1 *N* hydrochloric acid added, and the solution is diluted to 50 ml. with isopropyl alcohol. The absorbance of this dilution is measured as above.

SUMMARY

1. The physical properties of haloperidol, a new neuroleptic substance, are described.

2. Methods describing the preparation of multiple-dose solutions for oral administration, solutions for injection, and compressed tablets of haloperidol are presented.

3. Analytical procedures for the assay of haloperidol in its different formulations are developed.

4. Stability studies indicate that haloperidol is stable in its dosage forms over considerable periods of time.

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Notes

Synthesis of Radioactive Cl^{36}-Dichloromethotrexate

By VINCENT T. OLIVERIO and JACK D. DAVIDSON

The role of folic acid in cellular metabolism (1) and the mechanism of action of folic acid antagonists (2) have been the subject of numerous studies for more than a decade. While limited quantities of C^{14}-labeled folic acid have been synthesized (3, 4) for tracer studies, to our knowledge, no published reports of such studies have been carried out with radioactive labeled folic acid antagonists. The demonstration of apparent growth inhibitory properties of dichloromethotrexate[1] (DCM) in both animal tumor (5) and microbiological (6) systems offered the possibility of labeling this folic acid antagonist with radioactive chlorine. We now wish to report a successful synthesis of sufficient Cl^{36}-DCM in good yield and high purity for tracer studies.

The preparation of Cl^{36}-DCM was accomplished by a simple modification of the method described by Angier, *et al.* (7), who employed a large excess of chlorine (6.5 moles per mole of methotrexate) in order to prevent formation of monochloromethotrexate. However, to utilize the Cl^{36} from hydrochloric acid-Cl^{36} best, dimethylformamide was substituted for formamide to exclude the possible chlorination of formamide. As a result of this modification only 2.2 moles of Cl_2^{36}, obtained by hydrogen peroxide oxidation of hydrochloric acid-Cl^{36}, were required. The yield of DCM was 50% with 97% radiochemical purity.

PROCEDURE

The apparatus train consisted of a gas generator flask fitted with a dropping funnel, a reaction flask, and a sodium hydroxide gas trap connected in this order by polyethylene (PE 300) tubing. Radioactive hydrochloric acid (1.6 *N*, 7.85 ml., 250 μc.-Cl^{36}) was neutralized in the generator flask with 4 *N* sodium hydroxide. The sodium chloride-Cl^{36} was evaporated to dryness and suspended in 3 ml. of 30% hydrogen peroxide. Ten milliliters of ice-cold 15% fuming sulfuric acid, under 2.5 psi nitrogen pressure in the dropping funnel, was added dropwise to the generator flask. A slow, steady evolution of chlo-

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[1] N - [3,5 - dichloro - 4 - [(2,4 - diamino - 6 - pteridinylmethyl)methylamino]benzoyl-Cl^{36}]glutamic acid is the full chemical name. The abbreviated generic name, DCM, is employed throughout the text.
The hydrochloric acid-Cl^{36} was obtained from the Oak Ridge National Laboratory on allotment of the Atomic Energy Commission.

rine was maintained by the rates of addition of acid, of shaking, and of cooling. The chlorine was bubbled into a stirred (magnetic bar) ice-cold solution of 1.28 Gm. (2.82 m*M*) of methotrexate in 10 ml. of dimethylformamide. The effluent gas from this reaction was conducted through a trap containing 10 ml. of 1 *N* sodium hydroxide. Following the addition of the sulfuric acid to the gas generator the apparatus train was slowly flushed with nitrogen while the chlorine generating mixture was heated to boiling to complete the transfer of chlorine. Finally, the reaction flask was clamped off from the reaction train and stirred in the cold for one hour. In subsequent operations all mother liquors and washes were saved for radioactive assay.

The reaction mixture was poured into 100 ml. of ice-cold water and complete precipitation of crude DCM was achieved by adjusting the solution to pH 3 with 1 Gm. of sodium acetate. The precipitate was filtered and washed with water, acetone, and ether. The crude product, 1.1 Gm., was dissolved in 75 ml. of 2% sodium bicarbonate solution, heated to 75°, treated with Darco G-60 charcoal, and filtered while hot through a Celite pad on a No. 42 Whatman filter paper circle in a sintered-glass funnel. The clear yellow filtrate was reheated to 75° and approximately 2 Gm. of anhydrous magnesium sulfate added to precipitate the magnesium salt of DCM. The salt was filtered, washed with water, and dissolved in 100 ml. of water at 75°. The turbid solution was filtered hot to give a clear light-yellow colored filtrate. The warm (50°) filtrate was acidified with 1 ml. of glacial acetic acid to precipitate DCM. The pure product was filtered, washed with water, and dried at room temperature *in vacuo*. The yield was 726 mg. (49%).

The distribution of the chlorine[36] in this synthesis was determined by the liquid scintillation counting of 0.2-ml. aliquots of the various fractions, dissolved in 18 ml. of 30% methanol in toluene containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-di[2-(5-phenyloxazoyl)] benzene. The absolute counting efficiency was 85%. The chlorine generation, transfer, and absorption was highly efficient with only 0.08% remaining in the generator solution and less than 0.001% escaping the reaction flask to be absorbed in the alkali trap. The reaction mother liquor contained 68% of the chlorine. Another 8% appeared in the pooled water washes of the crude DCM and 4% in the acetone wash.

The pure radioactive product had a specific activity of 37 μc./m*M* and accounted for 20% of the chlorine[36] employed. The radiopurity of the product was assessed by paper chromatography and high voltage paper electrophoresis followed by autoradiography and counting of the radioactivity in all spots. Using ascending chromatography in sodium acetate buffer at pH 5.9, 97% of the radioactivity appeared at R_f 0.46 with 3% in a separate spot at R_f 0.12. In a descending system of 5% ammonium sulfate solution, isopropanol, and water (40:1:10), DCM had an R_f of 0.29 and a radioactive contaminant amounting to 1–2% appeared at R_f 0.36. Only a single radioactive spot appeared after high voltage electrophoresis at pH 7. At pH 3.5 the radioactive DCM migrated toward the cathode while 3% of the radioactivity moved an equal distance toward the anode and had the same mobility as authentic N^{10}-methyldichloropteroylglutamic acid. Chromatography of DCM on DEAE as previously described (8) also confirmed the 97% purity of the product. Finally, the radioactive DCM was in spectral (ultraviolet absorption) agreement with the data for DCM reported by Angier, *et al.* (7).

The 3% N^{10}-methyldichloropteroylglutamic acid contaminating our product probably arose from N^{10}-methylpteroylglutamic acid (methopterin) which is a common contaminant of methotrexate (9, 10) and was known to be present in the methotrexate employed in this synthesis. The purity of the DCM can be improved by chromatography on DEAE cellulose (8) but this appears unnecessary for studies concerned with elucidating the fate of the vast bulk, 97%, of doses of the compound. Such pharmacological studies in animals and humans are currently in progress.

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Adsorption of Phenothiazine Derivatives by Kaolin, Talc, and Norit

By DONALD L. SORBY† and ELMER M. PLEIN

Adsorption of chlorpromazine hydrochloride by wool, erythrocytes, and tissue was reported by Besson, *et al.* (1), and adsorption of phenothiazine derivatives by fritted-glass filters has been observed in our laboratory.

In view of these observations, it was believed that adsorption of phenothiazine derivatives by intestinal adsorbents or certain pharmaceutic aids might occur to a significant extent. Since this adsorption would be of considerable pharmaceutical interest, an investigation of the problem was initiated. As the first part of this study, the extent of adsorption of hydrochloride salts of 15 phenothiazine derivatives by kaolin, talc, and Norit A, an activated carbon, was measured.

The method used to determine the adsorption isotherms was similar to that of Evcim and Barr (2). To a mixture of 100 ml. of distilled water and the weighed amount of adsorbent contained in an amber 250-ml. glass-stoppered bottle was added the desired quantity of a 1×10^{-2} *M* solution of the phenothiazine derivative in the hydrochloride salt form.[1] Amounts of solution added varied from 2 to 15 ml. for talc and kaolin and from 7 to 25 ml. for Norit A. The preparations were shaken in a constant temperature bath at 20° for a minimum of four hours for kaolin and ten hours for talc and Norit A. The samples were then centrifuged for a maximum of twenty minutes, temperatures of the samples after centrifuging were within 20 ±1°. The concentration of phenothiazine derivative in the supernate was determined by a spectrophotometric method using a background cancellation technique patterned after the procedure of Flanagan, *et al.* (3), for chlorpromazine. The quantity of phenothiazine derivative adsorbed was calculated from the difference between the amount initially added to the contents of the bottle and the amount remaining in solution at equilibrium. No differences in concentration were noted in samples to which adsorbent had not been added.

Ethopropazine hydrochloride appeared to decompose after contact with talc and Norit A. The ultraviolet spectrum of the compound in solution after equilibration with the adsorbent showed a spectral shift toward the shorter wavelengths. This probably indicates oxidation to the 5-sulfoxide form. No spectral data were available for ethopropazine 5-sulfoxide; however, Flanagan, *et al.* (3), reported a spectrum for chlorpromazine 5-sulfoxide in which absorption peak shifts from unoxidized to oxidized material were very similar to the spectral shifts encountered here with ethopropazine. No such spectra changes were found for any of the other phenothiazine derivatives following equilibration with adsorbents.

The remaining data, with exception of dibasic compounds adsorbed on talc, gave linear plots according to the Langmuir equation. The value of the Langmuir constant, k_2, determined from the slope of a regression line calculated by the method of least squares from the experimental data was used to measure the extent of adsorption at saturation of the adsorbent surface. The estimated error of the value of k_2 was ±5%.

In all cases the amount of phenothiazine derivative adsorbed was considerable. The results are summarized in Table I.

Table I.—Adsorption of Phenothiazine Derivatives from Unbuffered Solution at 20°[a]

Compound	$mM \times 10^3$ Adsorbed by Kaolin, 1 Gm.	$mM \times 10^3$ Adsorbed by Talc, 1 Gm.	$mM \times 10^3$ Adsorbed by Norit A, 0.1 Gm.
Methoxypromazine hydrochloride	12.0	21.0	75.2
Pyrathiazine hydrochloride	18.9	39.7	101.0
Triflupromazine hydrochloride	19.3	61.3	80.0
Acepromazine hydrochloride	21.8	35.2	90.9
Trifluoperazine dihydrochloride	14.5	..[b]	65.8
Trimeprazine hydrochloride	23.0	47.8	91.7
Thiopropazate dihydrochloride	14.1	..[b]	58.5
Chlorpromazine hydrochloride	24.0	51.8	102.0
Promethazine hydrochloride	22.4	44.8	103.1
Promazine hydrochloride	21.9	49.5	116.3
Prochlorperazine dihydrochloride	15.2	..[b]	81.3
Fluphenazine dihydrochloride	15.1	..[b]	64.9
Mepazine hydrochloride	26.2	52.9	91.7
Thioridazine hydrochloride	35.9	74.1	72.5
Ethopropazine hydrochloride	24.0	..[c]	...[c]

[a] Obtained from the value of the Langmuir constant, k_2.
[b] The data plotted according to the Langmuir equation gave a nonlinear curve for the adsorption of dibasic compounds by talc.
[c] Evidence indicated a decomposition of the sample with these adsorbents.

The study is being extended to investigate the mechanism and factors which influence the adsorption process. Possible applications of the adsorption phenomena are also under investigation. Results of these studies will be reported at a later date.

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[1] Compounds not commercially available as hydrochloride salts were converted to this form using an anion exchange resin, Amberlite IRA-400.

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Antibiotic Properties of *Sargassum natans* from Puerto Rico

By NOEMÍ G. MARTÍNEZ NADAL

SEVERAL INVESTIGATORS have found that extracts of certain species of marine algae collected along the central California coast (1, 2), off the coast of the British Isles (3), and elsewhere (4) inhibit the growth of several species of Gram-positive and Gram-negative bacteria *in vitro*. In some of the studies it was shown that the antibacterial activity was not due to the presence of iodine (1, 2).

This note adds *Sargassum natans* to the list of species previously reported by others to possess antibacterial properties.

EXPERIMENTAL PROCEDURE

Sargassum natans, dried to reduce its moisture content to 12.82–16.15%, was extracted with ether in a Soxhlet apparatus. The extract was concentrated to a volume such that 0.1 ml. was equivalent to 0.2 Gm. of dried alga. *Penicillium notatum* was used as a comparative antibiotic organism, and extracts of it were prepared and tested in the same manner as the algal extracts. Ether controls with the same volume content were run as blanks through the routine extraction and assay of each test.

Assays were performed following the method of Sherwood (5). Sterile filter paper disks (diam., 15 mm.) containing 0.1 ml. of concentrated extract were placed on agar Petri plates (diam., 90 mm.), seeded with *Bacillus subtilis*, and the plates incubated for eighteen hours at 37°.

Received March 17, 1960, from the Research Center, College of Agriculture and Mechanic Arts, University of Puerto Rico.

Mrs. Carmen A. de Navarro, Scientific Aide, carried out the routine work involved.

RESULTS

No inhibition of growth occurred on control plates (disks without addition and disks with ether blank), clear zones (26 mm. radius) were present on plates with the *Penicillium* extract, and there was complete inhibition (no growth) on plates with the algal extract, even when the observation period was extended to seventy-two hours. Since the algal extracts did not permit visible growth to occur, it seemed desirable to ascertain whether they could cause lysis of existing organisms. Accordingly, 25 μL. of *S. natans* extract was added to the control disks (previously carrying no liquid) which were completely surrounded by bacterial growth. After twenty-four hours, well defined clear zones (radius, 22 mm.) were apparent. Subsequent sextuplicate tests in which 25 μL. of extract was placed on each assay disk on seeded plates resulted in clearly defined zones ranging from 19 mm. to 34 mm., and averaging 26.6 mm. in radius, after eighteen hours of incubation.

CONCLUSIONS

These preliminary tests indicate that an ether extract of *Sargassum natans* exhibits antibiotic properties against *Bacillus subtilis* and that the activity of the extracts compares favorably with that of extracts prepared in the same way from *Penicillium notatum*.

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Book Notices

Tobacco. Experimental and Clinical Studies. Edited by P. S. LARSON, H. B. HAAG, and H. SILVETTE. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1961. xii + 932 pp. 21.5 × 28 cm. Price $20.

A comprehensive review of information gleaned from more than 6,000 articles published in journals in many countries up to and into the year 1959. The monograph is divided into 22 coordinated chapters which cover every facet of research relating to tobacco. It is an excellent reference volume. Credits to original authors are indicated by name and year of publication throughout the text, with an alphabetically arranged bibliography (110 pages) appended. A subject index also is included.

Radioisotopes and Nuclear Techniques in the Pharmaceutical and Allied Industries. By JOHN E. CHRISTIAN and WILLIAM F. BOUSQUET. School of Pharmacy, Bionucleonics Dept., Purdue University, Lafayette, Ind., 1960. 119 pp. 21.5 × 28 cm. Paperbound.

The current and potential uses of radioisotopes and nuclear techniques in the pharmaceutical and allied industries are discussed in this report, which is based upon data obtained from 113 pharmaceutical companies. Much practical and useful information for anyone considering the use of "hot" procedures is included. [Editor: A supplementary review article by the senior author appeared in THIS JOURNAL, **50**, 1(1961).]

Amino Acides, Peptides, and Protéines. Vol. 4. Techniques et Travaux Expérimentaux. Edited by MAURICE VIGNERON. Societe de Chimie Industrielle, 28, Rue Saint-Dominique, Paris 7e, 1960. 294 pp. 21×27 cm. Paperbound.

A brief general introduction and a discussion on the synthesis of methionine are followed by two main divisions of the text of this book (in French). Part 1—Analysis: covers generalities, chemical and microbiological determinations, and paper chromatography. Part 2—Biology: covers metabolic and functional studies, and studies on tolerance and nutrition.

Clinical Disorders of the Pulmonary Circulation. Edited by RAYMOND DALEY, JOHN F. GOODWIN, and ROBERT E. STEINER. Medical Book Department, Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1960. xii+364 pp. 19×25 cm. Price $14.

Designed primarily for senior medical students and clinicians concerned with the diagnosis and treatment of disorders of the heart and the pulmonary circulation, this book contains the following chapters contributed by selected British practitioners: Anatomy of the pulmonary vasculature, Physiology and hemodynamics, Pulmonary function and circulation, The nature of pulmonary hypertension, The radiology of the pulmonary circulation, Pathology, Electrocardiography, Mitral valve and left atrial disease, Congenital heart disease, The pulmonary hyaline membrane syndrome of the newborn, Chronic pulmonary heart disease (chronic cor pulmonale), Chronic pulmonary disease, Pulmonary vascular obstruction, Pulmonary oedema. A general index is appended.

Chromatographic and Electrophoretic Techniques. Vol. 1, Chromatography. Vol. 2, Zone Electrophoresis. Edited by IVOR SMITH. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. Vol. 1, xvii+617 pp. Vol. 2, viii+215 pp. 14×21.5 cm. Price Vol. 1, $10.75; Vol. 2, $5.50.

Volume 1 of this 2-volume set describes in detail a single chromatographic apparatus, a minimal number of solvents and reagents, with tables of color reactions and R_f values. The factors affecting separation are discussed, and applications of the method are described. Volume 2 treats similarly the subject of zone electrophoresis. Both volumes stress practical methods of application for the techniques discussed. Volume 1 includes an index of compounds in the tabulations, and both volumes have general indexes.

Haemopoiesis. Cell Production and Its Regulation. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1960. xii+490 pp. 13.5×20 cm. Price $11.

Reports on 16 subjects related to hemopoiesis, together with discussions, comprise the proceedings of the 60th Ciba Foundation Symposium held February 1960. Author and subject indexes are appended.

ACTH. Edited by H. F. WEST. Munksgaard International Booksellers and Publishers Ltd., Nørregade 6, Copenhagen, Denmark, 1960. 135 pp. 15.5×23.5 cm. Paperbound.

The reports and discussions at the Sheffield Corticotropin Conference, held January 1960, are compiled in this publication. The 10 reports covered the nature of corticotropins, their bioassay, pharmacologic behavior, and some therapeutic applications.

Organic Syntheses. Vol. 40. Edited by MELVIN S. NEWMAN. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. vii+114 pp. 15×24 cm. Price $4.

Included in volume 40 of this classic series are: 3-acetyloxindole, 2-aminofluorene, 3-bromoacetophenone, 3-chloro-2,2,3-trifluoropropionic acid, 3-cyclohexenone, deoxyanisoin, 4,4′-diaminoazobenzene, diethyl acetamidomalonate, diethyl aminomalonate hydrochloride, diethyl bis(hydroxymethyl) malonate, 2,5-dimethoxy-2,5-dihydrofuran, N,N-dimethylaminomethylferrocene methiodide, 2,4-dinitroiodobenzene, 1,4-diphenyl-1,3-butadiene, α-β-diphenylpropionic acid, 3-ethoxy-2-cyclohexenone, ethyl 6,7-dimethoxy-2-methylindene-2-carboxylate, ferrocenylacetonitrile, 2-(2-furyl)acrylonitrile, 2-hydroxyisophthalic acid, hydroxymethylferrocene, 3-hydroxyquinoline, hydroxyurea, methanesulfinyl chloride, methylenecyclohexane, 1-methylindole, 2,6-naphthalenedicarboxylic acid, N-β-Naphthylpiperidine, Neopentyl alcohol, *m*-nitrophenyl disulfide, N-phthalyl-L-β-phenylalanine, *p*-Quinoquephenyl, 2,2,5,5-tetramethyltetrahydro-3-ketofuran, α,α,α′,α′-tetramethyltetramethylene glycol, *p*-toluenesulfonylhydrazide, 2,4,5-triaminonitrobenzene, 1,2,4-triazole, α,α,α-trichloroacetanilide, triphenylene. A subject index is appended.

Pharmacology. The Nature, Action and Use of Drugs. 2nd ed. By HARRY BECKMAN. W. B. Saunders Co., West Washington Square, Philadelphia 5, Pa., 1961. xvii + 805 pp. 18 × 25 cm. Price $15.50.

Described as a "textbook for the undergraduate medical student who can devote but a fleeting moment in his career to prepare for the pharmacologic aspects of the practice that lies ahead," this second edition of Dr. Beckman's book is an excellent reference for many basic and lasting concepts in this special area. The text is written in the cogent, concise, and readable style associated with the author. One might disagree with the decision to teach the calculations for prescribing teaspoon doses based on a volume of 4.8 ml. per teaspoon. This was determined by the author's past experience with various teaspoons and cannot be refuted. However, the U. S. P. XVI and N. F. XI have suggested the value of 5 ml. per teaspoon based upon the volume (4.93 ± 0.24 ml.) assigned by the American Standards Association to teaspoons that are being manufactured. To have one group of physicians (Beckman's students) using 4.8 ml. while practically all others are using 5 ml. as the volume for teaspoon dosage calculations could be unrealistic. Reference bibliographies are given after each chapter and a good index is appended.

Remington's Practice of Pharmacy. 12th ed. Edited by ERIC W. MARTIN and E. FULLERTON COOK. Mack Publishing Co., 20th and Northampton Sts., Easton, Pa., 1961. xii + 1896 pp. 20.5 × 28.5 cm. Price $22.50.

The extensive favorable review of R. P. P. XI which appeared in THIS JOURNAL, **45**, 192(1956), can be brought up to date and applied to R. P. P. XII. The major change in this revision is the arrangement of drugs (Section V, pages 685–1290) by pharmacological classification. Extensive revision is evident throughout the text and, as usual, part of the information in the latest editions of the U. S. P. and N. F. is incorporated. Ready association of trade names with generic names is an essential teaching aid which is continued. An alphabetical listing of the "Categories" assigned to the drugs in U. S. P. XVI and N. F. XI is included (Chapter 98, pages 1785–1795).

The excellence of the general discussion portions of the text is a reflection of the capable editors: E. W. Martin, E. F. Cook, E. E. Leuallen, A. Osol, L. F. Tice, and C. T. Van Meter, in addition to 56 assistant editors and about 200 contributors. The production work was well done by J. E. Hoover. This is a noteworthy addition to a classic series and should be in every pharmaceutical library. The thorough indexing and cross indexing adds to the value of this book as a reference source.

Evaluation of Drug Therapy. Edited by FRANCIS M. FORSTER. The University of Wisconsin Press, 430 Sterling Court, Madison 6, Wis., 1961. xxiv + 167 pp. 13.5 × 21.5 cm. Price $4.

Proceedings of a symposium on evaluation of drug therapy in neurologic and sensory diseases held at the University of Wisconsin, May 1960, are compiled in this book. Three papers and eight panel reports are included. A general index is appended.

Year Book of Drug Therapy 1960–1961. Edited by HARRY BECKMAN. The Year Book Publishers, Inc., 200 East Illinois St., Chicago 11, Ill., 1961. 659 pp. 13 × 19.5 cm. Price $8.50.

Thorough reviews of the medical literature for the year ending September 1960 are included in this issue of Dr. Beckman's annual publication. The book also includes "A critical evaluation of the year's new drugs." Indexes help this useful reference source.

A Short Textbook of Radiotherapy for Technicians and Students. By J. C. WALTER and H. MILLER. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1959. xi + 527 pp. 15 × 23 cm.

In this second edition (1959) of their book (1st ed. 1950) the authors have revised the material to include more recent developments in equipment and techniques utilized in radiotherapy. The text is based upon lectures to students, but the book should be a useful reference for radiographers. An appendix discusses radiation hazards in peace and war, and a general index is included.

Experimental Pharmaceutical Technology. By EUGENE L. PARROTT and WITOLD SASKI. Burgess Publishing Co., 426 South Sixth St., Minneapolis 15, Minn., 1961. iv + 256 pp. 21 × 27.5 cm. Paperbound. Price $5.75.

The authors' attempt in this manual to aid in the integration of a modern pharmaceutical preparations course with classical physical chemistry is logical and should be successful if the material is properly presented to students. The physical science concept of pharmacy is illustrated and explained by the exercises and experiments. All modern teachers of pharmacy and their students can benefit from this presentation.

New and Nonofficial Drugs 1961. Evaluated by the A. M. A. Council on Drugs. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1961. xxix + 849 pp. 12.5 × 18.5 cm. Price $3.35.

The 1961 compilation of available information on the drugs included in N. N. D. concisely and authoritatively describes their therapeutic, prophylactic, and diagnostic status as evaluated by the A. M. A. Council on Drugs. The value and usefulness of this annual publication are well established. It might be advisable to reconsider the title of the book because the proportion of official (U. S. P. and N. F.) drugs appears to be increasing. This is probably due to the rapid turnover of the materia medica in the five-year revisions of the official compendia, while the Council on Drugs bases its deletion policy on a twenty-year recognition period. Nevertheless, the information given in N. N. D. 1961 is so valuable that a new title for the book would be preferable to the omission of official drugs.

Medicine Makers of Kalamazoo. By LEONARD ENGEL. McGraw-Hill Book Co., Inc., 330 West 42nd St., New York 36, N. Y., 1961. viii + 261 pp. 13.5 × 20 cm. Price $4.50

An interesting review of the American pharmaceutical industry is related to the development and growth of the Upjohn Pill and Granule Company (1885) into the present Upjohn Company. An index is appended.

Textbook of Pharmacognosy. 4th ed. By T. E. WALLIS. J. & A. Churchill Ltd., London, 1960. Distributed in the United States by Little, Brown and Co., 34 Beacon St., Boston 6, Mass. xi + 640 pp. 13.5 × 21 cm. Price $10.

Revised and edited to include more recent knowledge of plant constituents, this book is a good working reference for pharmacognosists.

The Biotic Associations of Cockroaches. By LOUIS M. ROTH and EDWIN R. WILLIS. The Smithsonian Institution, Washington, D. C., 1960. vi + 470 pp. 15.5 × 23 cm. Paperbound.

A compilation of summarized material from approximately 1,700 source references. Thirty-seven pictures are included and a general index is appended. The book is a valuable reference on cockroaches.

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